RETROVIRAL VECTORS

This invention relates to a retroviral vector. In particular, but not exclusively, it relates to retroviral vectors capable of transferring genetic material to non-dividing or slowly-dividing cells derived from non-primate lentiviruses.

There has been considerable interest, for some time, in the development of retroviral vector systems based on lentiviruses, a small subgroup of the retroviruses. This interest arises firstly from the notion of using HIV-based vectors to target anti-HIV therapeutic genes to HIV susceptible cells and secondly from the prediction that, because lentiviruses are able to infect non-dividing cells (Lewis & Emerman 1993 J.Virol. 68, 510), vector systems based on these viruses would be able to transduce non-dividing cells (e.g. Vile & Russel 1995 Brit. Med. Bull. 51, 12). Vector systems based on HIV have been produced (Buchschacher & Panganiban 1992 J.Virol. 66, 2731) and they have been used to transduce CD4+ cells and, as anticipated, non-diving cells (Naldini *et al*, 1996 Science 272, 263). In addition lentiviral vectors enable very stable long-term expression of the gene of interest. This has been shown to be at least three months for transduced rat neuronal cells. The MLV based vectors were only able to express the gene of interest for six weeks.

HIV-based vectors produced to date result in an integrated provirus in the transduced cell that has HIV LTRs at its ends. This limits the use of these vectors as the LTRs have to be used as expression signals for any inserted gene unless an internal promoter is used. The use of internal promoters has significant disadvantages. The unpredictable outcome of placing additional promoters within the retroviral LTR transcription unit is well documented (Bowtell *et al*, 1988 J.Virol. 62, 2464; Correll *et al*, 1994 Blood 84, 1812; Emerman and Temin 1984 Cell 39, 459; Ghattas *et al*, 1991 Mol.Cell.Biol. 11, 5848; Hantzopoulos *et al*, 1989 PNAS 86, 3519; Hatzoglou *et al*, 1991 J.Biol.Chem 266, 8416; Hatzoglou *et al*, 1988 J.Biol.Chem 263, 17798; Li *et al*, 1992 Hum.Gen.Ther. 3, 381; McLachlin *et al*, 1993 Virol. 195, 1; Overell *et al*, 1988 Mol.Cell Biol. 8, 1803; Scharfman *et al*, 1991 PNAS 88, 4626; Vile *et al*, 1994 Gene Ther 1, 307; Xu *et al*, 1989 Virol. 171, 331; Yee *et al*, 1987 PNAS 84,

5197). The factors involved appear to include the relative position and orientation of the two promoters, the nature of the promoters and the expressed genes and any selection procedures that may be adopted. The presence of internal promoters can affect both the transduction titers attainable from a packaging cell line and the stability of the integrated vector.

HIV and other lentiviral LTRs have virus-specific requirements for gene expression. For example, the HIV LTR is not active in the absence of the viral Tat protein (Cullen 1995 AIDS 9, S19). It is desirable, therefore, to modify the LTRs in such a way as to change the requirements for gene expression. In particular tissue specific gene expression signals may be required for some gene therapy applications.

HIV vectors have a number of significant disadvantages which may limit their therapeutic application to certain diseases. HIV-1 has the disadvantage of being a human pathogen carrying potentially oncogenic proteins and sequences. There is the risk that introduction of vector particles produced in packaging cells which express HIV gag-pol will introduce these proteins into the patient leading to seroconversion. For these reasons, there is a need to develop lentiviral-based vectors which do not introduce HIV proteins into patients.

We have now found it possible to provide an improved lentiviral vector which overcomes the limitations of HIV-based vectors. It is important in the development of any retroviral vector system to remove sequences from the retroviral genome which may inhibit the capacity of the vector to transfer heterologous genes or which may transfer disadavantageous protein coding sequences to the target cell. Retroviruses are limited in the length of RNA sequences which can be packaged efficiently and so the existence of long regions of the retroviral genome severely limits the coding capacity of the vector for heterologous coding RNA.

We have also found it possible to provide a lentiviral vector based on a non-primate lentivirus which has a high coding capacity for heterologous coding sequences and which

has a reduced capacity to transfer retroviral components to the target cell.

It has surprisingly been found that the amount of vector genomic sequence required from a non-primate lentivirus to produce an efficient cloning vector is substantially less than has been described for an HIV-based vector.

The sequence requirements for packaging HIV vector genomes are complex. The HIV-1 packaging signal encompasses the splice donor site and contains a portion of the 5'-untranslated region of the *gag* gene which has a putative secondary structure containing 4 short stem-loops. Additional sequences elsewhere in the genome are also known to be important for efficient encapsidation of HIV. For example the first 350 bps of the gag protein coding sequence may contribute to efficient packaging and ill defined regions of env are also implicated. For the construction of HIV-vectors capable of expressing heterologous genes, a packaging signal extending to 350bps of the gag protein-coding region has been used.

We have surprisingly found that the structure of the packaging signal in non-primate lentiviruses is entirely different from that of HIV. Instead of a short sequence of 4 stem loops followed by an ill defined region of *gag* and *env* sequences, we have discovered that a shorter region of the *gag* gene suffices for efficient packaging. Indeed deletion of larger regions of the *gag* gene in EIAV vectors is advantageous and leads to higher titre viral vector being produced. This information can be used to provide improved vectors constructed from non-primate lentivirus sequences which have high titre and advantageous features compared to HIV vectors.

In a first aspect of the invention, there is provided a retroviral vector genome containing a deleted *gag* gene from a non-primate lentivirus wherein the deletion in *gag* removes one or more nucleotides downstream of nucleotide 350 of the gag coding sequence. Preferably the deletion extends from nucleotide 350 to at least the C-terminus of the *gagpol* coding region. More preferably the deletion additionally removes nucleotide 300 of the *gag*

coding region and most preferably the deletion retains only the first 150 nucleotides of the *gag* coding region. However even larger deletions of *gag* can also be used, for example the *gag* coding region contains the first 109 nucleotides of the *gag* coding region. It may also be possible for the *gag* coding region to contain only the first 2 nucleotides of the *gag* coding region.

Additional features of the lentiviral genome are included in the vector genome which are necessary for transduction of the target cell; replication; reverse transcription and integration. These are, at least, a portion of an LTR containing sequence from the R-region and U5 region, sequences from the 3' LTR which contain a polypurine tract (PPT) and a 3'LTR from the non-primate lentivirus or a hybrid LTR containing sequences from the non-primate lentivirus and other elements. Optionally, the retroviral genome may contain accessory genes derived from a retrovirus, such as, but not limited to, a *rev* gene, a *tat* gene, a *vif* gene, a *nef* gene, a *vpr* gene or an *S2* gene. Additional components may be added such as introns, splice-donor sites, a rev responsive element (RRE), cloning sites and selectable marker genes.

Moreover, we have now surprisingly demonstrated that a non-primate lentivirus minimal vector system can be constructed which requires neither S2, Tat, env nor dUTPase for either vector production or for transduction of dividing and non-dividing cells.

Thus according to another aspect the non-primate lentivirus genome from which the vector is derived lacks one or more accessory genes.

The deletion of accessory genes is highly advantageous. Firstly, it permits vectors to be produced without the genes normally associated with disease in lentiviral (e.g. HIV) infections. In particular, *tat* and *env* are associated with disease. Secondly, the deletion of accessory genes permits the vector to package more heterologous DNA. Thirdly, genes whose function is unknown, such as *dUTPase* and *S2*, may be omitted, thus reducing the risk of causing undesired effects.

In addition, we have shown that the leader sequence of the non-primate lentivirus genome is essential for high protein expression of *gag* and *gagpol*.

Therefore in a further aspect the non-primate lentivirus genome from which the vector is derived lacks the *tat* gene but includes the leader sequence between the end of the 5' LTR and the ATG of *gag*.

These data further define a minimal essential set of functional components for an optimal lentiviral vector. A vector is provided with maximal genetic capacity and high titre, but without accessory genes that are either of unknown function (*S2*, *UTPase*), and therefore may present risk, or are analogues of HIV proteins that may be associated with AIDS (*tat*, *env*).

It will be appreciated that the present invention provides a retroviral vector derived from a non-primate lentivirus genome (1) comprising a deleted *gag* gene wherein the deletion in gag removes one or more nucleotides downstream of nucleotide 350 of the *gag* coding sequence; (2) wherein one or more accessory genes are absent from the non-primate lentivirus genome; (3) wherein the non-primate lentivirus genome lacks the *tat* gene but includes the leader sequence between the end of the 5' LTR and the ATG of *gag*; and combinations of (1), (2) and (3). In a preferred embodiment the retroviral vector comprises all of features (1) and (2) and (3).

A "non-primate" vector, as used herein, refers to a vector derived from a virus which does not primarily infect primates, especially humans. Thus, non-primate virus vectors include vectors which infect non-primate mammals, such as dogs, sheep and horses, reptiles, birds and insects.

A lentiviral or lentivirus vector, as used herein, is a vector which comprises at least one component part derived from a lentivirus. Preferably, that component part is involved in

the biological mechanisms by which the vector infects cells, expresses genes or is replicated.

The non-primate lentivirus may be any member of the family of lentiviridae which does not naturally infect a primate and may include a feline immunodeficiency virus (FIV), a bovine immunodeficiency virus (BIV), a caprine arthritis encephalitis virus (CAEV), a Maedi visna virus (MVV) or an equine infectious anaemia virus (EIAV). Preferably the lentivirus is an EIAV. Equine infectious anaemia virus infects all equidae resulting in plasma viremia and thrombocytopenia (Clabough, et al. 1991. J Virol. 65:6242-51). Virus replication is thought to be controlled by the process of maturation of monocytes into macrophages.

EIAV has the simplest genomic structure of the lentiviruses. In addition to the *gag*, *pol* and *env* genes EIAV encodes three other genes: *tat*, *rev*, and *S2*. *Tat* acts as a transcriptional activator of the viral LTR (Derse and Newbold1993 Virology. 194:530-6; Maury, et al 1994 Virology. 200:632-42.) and Rev regulates and coordinates the expression of viral genes through rev-response elements (RRE) (Martarano et al 1994 J Virol. 68:3102-11.). The mechanisms of action of these two proteins are thought to be broadly similar to the analogous mechanisms in the primate viruses (Martano et al ibid). The function of S2 is unknown. In addition, an EIAV protein, Ttm, has been identified that is encoded by the first exon of *tat* spliced to the *env* coding sequence at the start of the transmembrane protein.

In addition to protease, reverse transcriptase and integrase non-primate lentiviruses contain a fourth *pol* gene product which codes for a dUTPase. This may play a role in the ability of these lentiviruses to infect certain non-dividing cell types.

The viral RNA in the first aspect of the invention is transcribed from a promoter, which may be of viral or non-viral origin, but which is capable of directing expression in a eukaryotic cell such as a mammalian cell. Optionally an enhancer is added, either

upstream of the promoter or downstream. The RNA transcript is terminated at a polyadenylation site which may be the one provided in the lentiviral 3' LTR or a different polyadenylation signal.

Thus the present invention provides a DNA transcription unit comprising a promoter and optionally an enhancer capable of directing expression of a retroviral vector genome.

Transcription units as described herein comprise regions of nucleic acid containing sequences capable of being transcribed. Thus, sequences encoding mRNA, tRNA and rRNA are included within this definition. The sequences may be in the sense or antisense orientation with respect to the promoter. Antisense constructs can be used to inhibit the expression of a gene in a cell according to well-known techniques. Nucleic acids may be, for example, ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or analogues thereof. Sequences encoding mRNA will optionally include some or all of 5' and/or 3' transcribed but untranslated flanking sequences naturally, or otherwise, associated with the translated coding sequence. It may optionally further include the associated transcriptional control sequences normally associated with the transcribed sequences, for example transcriptional stop signals, polyadenylation sites and downstream enhancer elements. Nucleic acids may comprise cDNA or genomic DNA (which may contain introns).

The term "promoter" is used in the normal sense of the art, e.g. an RNA polymerase binding site in the Jacob-Monod theory of gene expression.

The term "enhancer" includes a DNA sequence which binds to other protein components of the transcription initiation complex and thus facilitates the initiation of transcription directed by its associated promoter.

The promoter and enhancer of the transcription units encoding the first viral vector component are preferably strongly active, or capable of being strongly induced, in the

producer cell under conditions for production of the retroviral vector of the present invention and/or in primary target cells under conditions for production of the secondary viral vector. The promoter and enhancer of the transcription units encoding the second viral vector component are preferably strongly active, or capable of being strongly induced, in the target cells. The promoter and/or enhancer may be constitutively efficient, or may be tissue or temporally restricted in their activity. Examples of suitable tissue restricted promoters/enhancers are those which are highly active in tumour cells such as a promoter/enhancer from a MUC1 gene, a CEA gene or a 5T4 antigen gene. Examples of temporally restricted promoters/enhancers are those which are responsive to ischaemia and/or hypoxia, such as hypoxia response elements or the promoter/enhancer of a *grp78* or a *grp94* gene. One preferred promoter-enhancer combination is a human cytomegalovirus (hCMV) major immediate early (MIE) promoter/enhancer combination.

The LTRs may be altered in, for example, U3 (such as to obtain strong constitutive expression, inducible expression or tissue specific expression); R (such as to remove TAR stem loops); or U5 (such as to use enhanced non-U5 based polyadenylation signals, for example from the bovine growth hormone gene).

In one configuration the internal promoter cassette is reversed and a polyadenylation signal is placed downstream of the cassette.

In another embodiment the polyadenylation signal which is used contains at least one intron.

The vector of the present invention may make use of self-inactivating strategies. Self-inactivating retroviral vectors have been constructed by deleting the transcriptional enhancers or the enhancers and promoters in the U3 region of the 3' LTR. After one round of vector replication, these changes are copied into both the 5' and the 3' LTRs producing an inactive provirus. However, any promoters internal to the LTRs in such vectors will still be active. This strategy has been employed to eliminate effects of the

enhancers and promoters in the viral LTRs on transcription from internally placed genes. Such effects include increased transcription or suppression of transcription. This strategy can also be used to eliminate downstream transcription from the 3' LTR into genomic DNA. This is of particular concern in human gene therapy where it is of critical importance to prevent any activation of an endogenous oncogene.

Another type of self-inactivating vector has been constructed that has direct repeats flanking the packaging signal such that the packaging signal is frequently deleted during reverse transcription, producing virus defective for packaging. With sufficiently long direct repeats, a majority of resultant proviruses lose their packaging sequences. The rate of deletion could be increased to 100% by designing the vector so that packaging signal deletion reconstituted the *neo* marker nad be selecting the vector-infected cells in G418. This strategy may be particularly useful for gene therapy applications where any spread of the vector following gene transfer is undesirable.

In a further preferred embodiment of the first aspect of the invention, one or more nucleotides of interest (NOI) is introduced into the vector at the cloning site. Such therapeutic genes may be expressed from a promoter placed in the retroviral LTR or may be expressed from an internal promoter introduced at the cloning site.

Suitable NOI coding sequences include those that are of therapeutic and/or diagnostic application such as, but are not limited to: sequences encoding cytokines, chemokines, hormones, antibodies, engineered immunoglobulin-like molecules, a single chain antibody, fusion proteins, enzymes, immune co-stimulatory molecules, immunomodulatory molecules, anti-sense RNA, a transdominant negative mutant of a target protein, a toxin, a conditional toxin, an antigen, a tumour suppressor protein and growth factors, membrane proteins, vasoactive proteins and peptides, anti-viral proteins and ribozymes, and derivatives therof (such as with an associated reporter group). When included, such coding sequences may be typically operatively linked to a suitable

promoter, which may be a promoter driving expression of a ribozyme(s), or a different promoter or promoters.

The NOI coding sequence may encode a fusion protein or a segment of a coding sequence.

The retroviral vector of the present invention may be used to deliver a NOI such as a pro-drug activating enzyme to a tumour site for the treatment of a cancer. In each case, a suitable pro-drug is used in the treatment of the individual (such as a patient) in combination with the appropriate pro-drug activating enzyme. An appropriate pro-drug is administered in conjunction with the vector. Examples of pro-drugs include: etoposide phosphate (with alkaline phosphatase, Senter *et al* 1988 Proc Natl Acad Sci 85: 4842-4846); 5-fluorocytosine (with cytosine deaminase, Mullen *et al* 1994 Cancer Res 54: 1503-1506); Doxorubicin-N-p-hydroxyphenoxyacetamide (with Penicillin-V-Amidase, Kerr *et al* 1990 Cancer Immunol Immunother 31: 202-206); Para-N-bis(2-chloroethyl) aminobenzoyl glutamate (with carboxypeptidase G2); Cephalosporin nitrogen mustard carbamates (with β-lactamase); SR4233 (with P450 Reducase); Ganciclovir (with HSV thymidine kinase, Borrelli *et al* 1988 Proc Natl Acad Sci 85: 7572-7576); mustard pro-drugs with nitroreductase (Friedlos *et al* 1997 J Med Chem 40: 1270-1275) and Cyclophosphamide (with P450 Chen *et al* 1996 Cancer Res 56: 1331-1340).

The vector of the present invention may be delivered to a target site by a viral or a non-viral vector.

As it is well known in the art, a vector is a tool that allows or faciliates the transfer of an entity from one environment to another. By way of example, some vectors used in recombinant DNA techniques allow entities, such as a segment of DNA (such as a heterologous DNA segment, such as a heterologous cDNA segment), to be transferred into a target cell. Optionally, once within the target cell, the vector may then serve to

maintain the heterologous DNA within the cell or may act as a unit of DNA replication. Examples of vectors used in recombinant DNA techniques include plasmids, chromosomes, artificial chromosomes or viruses.

Non-viral delivery systems include but are not limted to DNA transfection methods. Here, transfection includes a process using a non-viral vector to deliver a gene to a target mammalian cell.

Typical transfection methods include electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection, liposomes, immunoliposomes, lipofectin, cationic agent-mediated, cationic facial amphiphiles (CFAs) (Nature Biotechnology 1996 14; 556), and combinations thereof.

Viral delivery systems include but are not limited to adenovirus vector, an adeno-associated viral (AAV) vector, a herpes viral vector, retroviral vector, lentiviral vector, baculoviral vector. Other examples of vectors include *ex vivo* delivery systems, which include but are not limited to DNA transfection methods such as electroporation, DNA biolistics, lipid-mediated transfection, compacted DNA-mediated transfection.

The term "retroviral vector particle" refers to the packaged retroviral vector, that is preferably capable of binding to and entering target cells. The components of the particle, as already discussed for the vector, may be modified with respect to the wild type retrovirus. For example, the Env proteins in the proteinaceous coat of the particle may be genetically modified in order to alter their targeting specificity or achieve some other desired function.

Preferably, the viral vector preferentially transduces a certain cell type or cell types.

More preferably, the viral vector is a targeted vector, that is it has a tissue tropism which is altered compared to the native virus, so that the vector is targeted to particular cells.

For retroviral vectors, this may be achieved by modifying the Env protein. The Env protein of the retroviral secondary vector needs to be a non-toxic envelope or an envelope which may be produced in non-toxic amounts within the primary target cell, such as for example a MMLV amphotropic envelope or a modified amphotropic envelope. The safety feature in such a case is preferably the deletion of regions or sequence homology between retroviral components.

Preferably the envelope is one which allows transduction of human cells. Examples of suitable *env* genes include, but are not limited to, VSV-G, a MLV amphotropic *env* such as the 4070A *env*, the RD114 feline leukaemia virus *env* or haemagglutinin (HA) from an influenza virus. The Env protein may be one which is capable of binding to a receptor on a limited number of human cell types and may be an engineered envelope containing targeting moieties. The *env* and *gag-pol* coding sequences are transcribed from a promoter and optionally an enhancer active in the chosen packaging cell line and the transcription unit is terminated by a polyadenylation signal. For example, if the packaging cell is a human cell, a suitable promoter-enhancer combination is that from the human cytomegalovirus major immediate early (hCMV-MIE) gene and a polyadenylation signal from SV40 virus may be used. Other suitable promoters and polyadenylation signals are known in the art.

The packaging cell may be an *in vivo* packaging cell in the body of an individual to be treated or it may be a cell cultured *in vitro* such as a tissue culture cell line. Suitable cell lines include mammalian cells such as murine fibroblast derived cell lines or human cell lines. Preferably the packaging cell line is a human cell line, such as for example: 293 cell line, HEK293, 293-T, TE671, HT1080.

Alternatively, the packaging cell may be a cell derived from the individual to be treated such as a monocyte, macrophage, stem cells, blood cell or fibroblast. The cell may be isolated from an individual and the packaging and vector components administered *ex vivo* followed by re-administration of the autologous packaging cells. Alternatively the

packaging and vector components may be administered to the packaging cell *in vivo*. Methods for introducing retroviral packaging and vector components into cells of an individual are known in the art. For example, one approach is to introduce the different DNA sequences that are required to produce a retroviral vector particle e.g. the *env* coding sequence, the *gag-pol* coding sequence and the defective retroviral genome into the cell simultaneously by transient triple transfection (Landau & Littman 1992 J. Virol. 66, 5110; Soneoka *et al* 1995 Nucleic Acids Res 23:628-633).

In one embodiment the vector configurations of the present invention use as their production system, three transcription units expressing a genome, the *gag-pol* components and an envelope. The envelope expression cassette may include one of a number of envelopes such as VSV-G or various murine retrovirus envelopes such as 4070A.

Conventionally these three cassettes would be expressed from three plasmids transiently transfected into an appropriate cell line such as 293T or from integrated copies in a stable producer cell line. An alternative approach is to use another virus as an expression system for the three cassettes, for example baculovirus or adenovirus. These are both nuclear expression systems. To date the use of a poxvirus to express all of the components of a retroviral or lentiviral vector system has not been described. In particular, given the unusual codon usage of lentiviruses and their requirement for RNA handling systems such as the rev/RRE system it has not been clear whether incorporation of all three cassettes and their subsequent expression in a vector that expresses in the cytoplasm rather than the nucleus is feasible. Until now the possibility remained that key nuclear factors and nuclear RNA handling pathways would be required for expression of the vector components and their function in the gene delivery vehicle. Here we describe such a system and show that lentiviral components can be made in the cytoplasm and that they assemble into functional gene delivery systems. The advantage of this system is the ease with which poxviruses can be handled, the high expression levels and the ability to retain introns in the vector genomes.

According to another aspect therefore there is provided a hybrid viral vector system for *in vivo* gene delivery, which system comprises a primary viral vector which is obtainable from or is based on a poxvirus and a second viral vector which is obtainable from or is based on a vectroviral vector, preferably a lentiviral vector, even more preferably a non-primate lentiviral vector.

The secondary vector may be produced from expression of essential genes for retroviral vector production encoded in the DNA of the primary vector. Such genes may include a *gag-pol* from a retrovirus, an *env* gene from an enveloped virus and a defective retroviral vector containing one or more therapeutic or diagnostic NOI(s). The defective retroviral vector contains in general terms sequences to enable reverse transcription, at least part of a 5' long terminal repeat (LTR), at least part of a 3'LTR and a packaging signal.

If it is desired to render the secondary vector replication defective, that secondary vector may be encoded by a plurality of transcription units, which may be located in a single or in two or more adenoviral or other primary vectors.

In some therapeutic or experimental situations it may be desirable to obviate the need to make EAIV derived from MVA in vitro. MVA-EIAV hybrids are delivered directly into the patient/animal e.g. MVA-EIAV is injected intravenously into the tail vein of a mouse and this recombinant virus infects a variety of murine tissues e.g. lung, spleen etc. Infected cells express transduction competent EIAV containing a therapeutic gene for gene therapy for example. EIAV vector particles bud from these cells and transduce neighbouring cells. The transduced cell then contains an integrated copy of the EIAV vector genome and expresses the therapeutic gene product or other gene product of interest. If expression of the therapeutic gene product is potentially toxic to the host it may be regulated by a specific promoter, e.g. the hypoxic response element (HRE), which will restrict expression to those cells in a hypoxic environment. For gene therapy of lung/trachea epithelium cells e.g to treat cystic fibrosis MVA-EIAV may be given as an aerosol delivered intranasally. Alternatively, macrophages can be transduced in vitro and

then reintroduced to create macrophage factories for EIAV-based vectors. Furthermore, because MVA is replication incompetent MVA-EIAV hybrids could also be used to treat immuno-suppressed hosts.

Vaccinia virus, the prototypic member of the orthopox genus within the family poxviridae, was the first virus used for expression of recombinant exogenous proteins (Mackett et al 1982, Paoletti & Panicalli 1982). Vaccinia virus has a large DNA genome of greater than 180 kb and reports indicate that it can accommodate over 25 kb of foreign DNA (Merchlinsky & Moss 1992). Several other strains of poxviruses have been adapted as recombinant expression vectors (for review see Carroll and Moss 1997) e.g. fowlpox (Taylor & Paoletti 1988), canarypox (Taylor et al 1991), swinepox (van der Leek et al 1994) and entomopox (Li et al 1997). Additionally, due to safety concerns, several highly attenuated strains of vaccinia virus have been developed that are compromised in human and other mammalian cells e.g. modified vaccinia virus Ankara (MVA) (Mayr 1978, Sutter 1992), NYVAC (Paoletti et al 1994), vaccinia virus deficient in a DNA replication enzyme (Holzer et al 1997). These may all be used in the present invention.

MVA was derived from a replication competent vaccinia smallpox vaccine strain, Ankara. After >500 passages in chick embryo fibroblast cells the virus isolate was shown to be highly attenuated in a number of animal models including mice that were immune deficient (Mayr et al 1978). The attenuated isolate, MVA, was used to vaccinate over 120,000 people, many of which were immunocompromised (Mahnel 1994) without adverse effects. Studies illustrate that MVA can infect a wide range of mammalian cells but productive infection has only been observed in Hamster kidney cell BHK-21 (Carroll 1997). In all other tested mammalian cell lines early expression, DNA replication and late expression are observed leading to the production of non-infectious immature virus particles (Carroll 1997, Meyer 1991). Virus replication studies show that a minority of mammalian cells can support very low level production of infectious virus i.e. BS-C-1 cells in which 1 infectious MVA particle is produced per cell (Carroll and Moss 1997). Late gene expression usually give rise to >10 fold more protein that those genes under

early promoters (Chakrabarti et al 1997, Wyatt et al 1996). In all other attenuated poxvirus strains late gene expression is rarely observed in mammalian cells.

Production of retrovirus vector systems e.g. MLV-HIV and lentivirus vector systems requires the construction of producer lines that express the virus genome and essential structural proteins to make transduction competent virus. Generally, this is a relatively inefficient process which is further complicated when the virus is pseudotyped with toxic envelope proteins such as VSV-G. Expression of a functional genome and the required structural proteins from within a recombinant poxvirus may obviate many of the current inefficient retrovirus and lentivirus vector production technologies. Additionally, such recombinant poxviruses may be directly injected into patients to give rise to *in vivo* production of retrovirus or lentivirus.

MVA is a particularly suitable poxvirus for the construction of a pox-retrovirus or pox-lentivirus hybrid due to its non-replicating phenotype and its ability to perform both early and strong late expression for the production of high titre vector preparations.

In order to produce a functional retrovirus or lentivirus vector genome it is essential that the 5' of the RNA genome should be exact (Cannon et al. 1996). This is a challenge in a vaccinia-based production system as many of the vaccinia promoter comprise downstream determinants of transcription efficiency (Davison 1989b, Moss 1996). However, we show that there are several ways to solve this problem:

a. Use of a T7 promoter and T7 termination sequence.

b. Use of early promoters (in which sequences downstream of the RNA start site are not highly conserved), (Davison 1989a).

c. Use of intermediate and late promoters of vaccinia which require additional sequences downstream of the initiation site in conjunction with strategies to generate an authentic 5' end or which place the additional downstream sequences into both R regions. There is a requirement for specific sequences of 4 nucleotides

downstream of the initiation of transcription in the late promoter (Davison 1989b, Moss 1996). In the first case a ribozyme is placed downstream of the promoter and upstream of the R region. The ribozyme is designed to cleave the RNA in cis to generate the correct 5' end. In the second the approach is to modify the R regions to incorporate the extra sequences. This must be done in both the 5' and the 3' LTR R regions.

The advantage of having a T7 dependent system is that it would require the infection of the cell by two recombinant vaccinia viruses to produce transducing EIAV viral particles. For example, one MVA could carry the vector genome, under the control of the T7 promoter and the *gag/pol* and the *env* sequences under the control of the vaccinia promoters. The other MVA would carry the T7 polymerase gene under the control of a vaccinia promoter (Wyatt et al 1995).

The retroviral vector particle according to the invention will also be capable of transducing cells which are slowly-dividing, and which non-lentiviruses such as MLV would not be able to efficiently transduce. Slowly-dividing cells divide once in about every three to four days including certain tumour cells. Although tumours contain rapidly dividing cells, some tumour cells especially those in the centre of the tumour, divide infrequently. Alternatively the target cell may be a growth-arrested cell capable of undergoing cell division such as a cell in a central portion of a tumour mass or a stem cell such as a haematopoietic stem cell or a CD34-positive cell. As a further alternative, the target cell may be a precursor of a differentiated cell such as a monocyte precursor, a CD33-positive cell, or a myeloid precursor. As a further alternative, the target cell may be a differentiated cell such as a neuron, astrocyte, glial cell, microglial cell, macrophage, monocyte, epithelial cell, endothelial cell or hepatocyte. Target cells may be transduced either *in vitro* after isolation from a human individual or may be transduced directly *in vivo*.

The delivery of one or more therapeutic genes by a vector system according to the present invention may be used alone or in combination with other treatments or components of the treatment.

For example, the retroviral vector of the present invention may be used to deliver one or more NOI(s) useful in the treatment of the disorders listed in WO-A-98/05635. For ease of reference, part of that list is now provided: cancer, inflammation or inflammatory disease, dermatological disorders, fever, cardiovascular effects, haemorrhage, coagulation and acute phase response, cachexia, anorexia, acute infection, HIV infection, shock states, graft-versus-host reactions, autoimmune disease, reperfusion injury, meningitis, migraine and aspirin-dependent anti-thrombosis; tumour growth, invasion and spread, angiogenesis, metastases, malignant, ascites and malignant pleural effusion; cerebral ischaemia, ischaemic heart disease, osteoarthritis, rheumatoid arthritis, osteoporosis, asthma, multiple sclerosis, neurodegeneration, Alzheimer's disease, atherosclerosis, stroke, vasculitis, Crohn's disease and ulcerative colitis; periodontitis, gingivitis; psoriasis, atopic dermatitis, chronic ulcers, epidermolysis bullosa; corneal ulceration, retinopathy and surgical wound healing; rhinitis, allergic conjunctivitis, eczema, anaphylaxis; restenosis, congestive heart failure, endometriosis, atherosclerosis or endosclerosis.

In addition, or in the alternative, the retroviral vector of the present invention may be used to deliver one or more NOI(s) useful in the treatment of disorders listed in WO-A-98/07859. For ease of reference, part of that list is now provided: cytokine and cell proliferation/differentiation activity; immunosuppressant or immunostimulant activity (e.g. for treating immune deficiency, including infection with human immune deficiency virus; regulation of lymphocyte growth; treating cancer and many autoimmune diseases, and to prevent transplant rejection or induce tumour immunity); regulation of haematopoiesis, e.g. treatment of myeloid or lymphoid diseases; promoting growth of bone, cartilage, tendon, ligament and nerve tissue, e.g. for healing wounds, treatment of burns, ulcers and periodontal disease and neurodegeneration; inhibition or activation of follicle-stimulating hormone (modulation of fertility); chemotactic/chemokinetic activity

(e.g. for mobilising specific cell types to sites of injury or infection); haemostatic and thrombolytic activity (e.g. for treating haemophilia and stroke); antiinflammatory activity (for treating e.g. septic shock or Crohn's disease); as antimicrobials; modulators of e.g. metabolism or behaviour; as analgesics; treating specific deficiency disorders; in treatment of e.g. psoriasis, in human or veterinary medicine.

In addition, or in the alternative, the retroviral vector of the present invention may be used to deliver one or more NOI(s) useful in the treatment of disorders listed in WO-A-98/09985. For ease of reference, part of that list is now provided: macrophage inhibitory and/or T cell inhibitory activity and thus, anti-inflammatory activity; anti-immune activity, i.e. inhibitory effects against a cellular and/or humoral immune response, including a response not associated with inflammation; inhibit the ability of macrophages and T cells to adhere to extracellular matrix components and fibronectin, as well as up-regulated fas receptor expression in T cells; inhibit unwanted immune reaction and inflammation including arthritis, including rheumatoid arthritis, inflammation associated with hypersensitivity, allergic reactions, asthma, systemic lupus erythematosus, collagen diseases and other autoimmune diseases, inflammation associated with atherosclerosis, arteriosclerosis, atherosclerotic heart disease, reperfusion injury, cardiac arrest, myocardial infarction, vascular inflammatory disorders, respiratory distress syndrome or other cardiopulmonary diseases, inflammation associated with peptic ulcer, ulcerative colitis and other diseases of the gastrointestinal tract, hepatic fibrosis, liver cirrhosis or other hepatic diseases, thyroiditis or other glandular diseases, glomerulonephritis or other renal and urologic diseases, otitis or other oto-rhino-laryngological diseases, dermatitis or other dermal diseases, periodontal diseases or other dental diseases, orchitis or epididimo-orchitis, infertility, orchidal trauma or other immune-related testicular diseases, placental dysfunction, placental insufficiency, habitual abortion, eclampsia, pre-eclampsia and other immune and/or inflammatory-related gynaecological diseases, posterior uveitis, intermediate uveitis, anterior uveitis, conjunctivitis, chorioretinitis, uveoretinitis, optic neuritis, intraocular inflammation, e.g. retinitis or cystoid macular oedema, sympathetic ophthalmia, scleritis, retinitis pigmentosa, immune and inflammatory components of

degenerative fondus disease, inflammatory components of ocular trauma, ocular inflammation caused by infection, proliferative vitreo-retinopathies, acute ischaemic optic neuropathy, excessive scarring, e.g. following glaucoma filtration operation, immune and/or inflammation reaction against ocular implants and other immune and inflammatory-related ophthalmic diseases, inflammation associated with autoimmune diseases or conditions or disorders where, both in the central nervous system (CNS) or in any other organ, immune and/or inflammation suppression would be beneficial, Parkinson's disease, complication and/or side effects from treatment of Parkinson's disease, AIDS-related dementia complex HIV-related encephalopathy, Devic's disease, Sydenham chorea, Alzheimer's disease and other degenerative diseases, conditions or disorders of the CNS, inflammatory components of stokes, post-polio syndrome, immune and inflammatory components of psychiatric disorders, myelitis, encephalitis, subacute sclerosing pan-encephalitis, encephalomyelitis, acute neuropathy, subacute neuropathy, chronic neuropathy, Guillaim-Barre syndrome, Sydenham chora, myasthenia gravis, pseudo-tumour cerebri, Down's Syndrome, Huntington's disease, amyotrophic lateral sclerosis, inflammatory components of CNS compression or CNS trauma or infections of the CNS, inflammatory components of muscular atrophies and dystrophies, and immune and inflammatory related diseases, conditions or disorders of the central and peripheral nervous systems, post-traumatic inflammation, septic shock, infectious diseases, inflammatory complications or side effects of surgery, bone marrow transplantation or other transplantation complications and/or side effects, inflammatory and/or immune complications and side effects of gene therapy, e.g. due to infection with a viral carrier, or inflammation associated with AIDS, to suppress or inhibit a humoral and/or cellular immune response, to treat or ameliorate monocyte or leukocyte proliferative diseases, e.g. leukaemia, by reducing the amount of monocytes or lymphocytes, for the prevention and/or treatment of graft rejection in cases of transplantation of natural or artificial cells, tissue and organs such as cornea, bone marrow, organs, lenses, pacemakers, natural or artificial skin tissue.

The present invention also provides a pharmaceutical composition for treating an individual by gene therapy, wherein the composition comprises a therapeutically effective amount of the retroviral vector of the present invention comprising one or more deliverable therapeutic and/or diagnostic NOI(s) or a viral particle produced by or obtained from same. The pharmaceutical composition may be for human or animal usage. Typically, a physician will determine the actual dosage which will be most suitable for an individual subject and it will vary with the age, weight and response of the particular individual.

The composition may optionally comprise a pharmaceutically acceptable carrier, diluent, excipient or adjuvant. The choice of pharmaceutical carrier, excipient or diluent can be selected with regard to the intended route of administration and standard pharmaceutical practice. The pharmaceutical compositions may comprise as - or in addition to - the carrier, excipient or diluent any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s), and other carrier agents that may aid or increase the viral entry into the target site (such as for example a lipid delivery system).

Where appropriate, the pharmaceutical compositions can be administered by any one or more of: inhalation, in the form of a suppository or pessary, topically in the form of a lotion, solution, cream, ointment or dusting powder, by use of a skin patch, orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents, or they can be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. For parenteral administration, the compositions may be best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood. For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

The delivery of one or more therapeutic genes by a vector system according to the invention may be used alone or in combination with other treatments or components of the treatment. Diseases which may be treated include, but are not limited to: cancer, neurological diseases, inherited diseases, heart disease, stroke, arthritis, viral infections and diseases of the immune system. Suitable therapeutic genes include those coding for tumour suppressor proteins, enzymes, pro-drug activating enzymes, immunomodulatory molecules, antibodies, engineered immunoglobulin-like molecules, fusion proteins, hormones, membrane proteins, vasoactive proteins or peptides, cytokines, chemokines, anti-viral proteins, antisense RNA and ribozymes.

In a preferred embodiment of a method of treatment according to the invention, a gene encoding a pro-drug activating enzyme is delivered to a tumour using the vector system of the invention and the individual is subsequently treated with an appropriate pro-drug. Examples of pro-drugs include etoposide phosphate (used with alkaline phosphatase Senter et al., 1988 Proc. Natl. Acad. Sci. 85: 4842-4846); 5-fluorocytosine (with Cytosine deaminase Mullen et al. 1994 Cancer Res. 54: 1503-1506); Doxorubicin-N-p-hydroxyphenoxyacetamide (with Penicillin-V-Amidase (Kerr et al. 1990 Cancer Immunol. Immunother. 31: 202-206); Para-N-bis(2-chloroethyl) aminobenzoyl glutamate (with Carboxypeptidase G2); Cephalosporin nitrogen mustard carbamates (with b-lactamase); SR4233 (with P450 Reducase); Ganciclovir (with HSV thymidine kinase, Borrelli et al. 1988 Proc . Natl. Acad. Sci. 85: 7572-7576) mustard pro-drugs with nitroreductase (Friedlos et al. 1997J Med Chem 40: 1270-1275) and Cyclophosphamide or Ifosfamide (with a cytochrome P450 Chen et al. 1996 Cancer Res 56: 1331-1340).

In accordance with the invention, standard molecular biology techniques may be used which are within the level of skill in the art. Such techniques are fully described in the literature. See for example; Sambrook *et al* (1989) Molecular Cloning; a laboratory manual; Hames and Glover (1985 - 1997) DNA Cloning: a practical approach, Volumes I-IV (second edition); Methods for the engineering of immunoglobulin genes are given in McCafferty *et al* (1996) "Antibody Engineering: A Practical Approach".

The invention will now be further described by way of example in which reference is made to the following Figures in which:

Figure 1 – shows the structure of transcription units from plasmids pESP, pONY3 and pONY2.1nlsLacZ.

Figure 2 – shows a PCR analysis of integrated EIAV vector. PCR was performed with either genomic DNA from EIAV vector transduced cells (lanes 1 and 5) or mock transduced cells (lanes 2 and 6). pONY2.1nlsLacZ (lanes 3 and 7) and pONY3 (lanes 4 and 8) were used as controls. A. PCR detection of EIAV LTRs. B. PCR detection of *pol*.

Figure 3 - shows the structure of vector transcription units in deletion plasmids used to identify the packaging requirements for an EIAV vector.

Figure 4 – shows a secondary structure prediction for the RNA derived from the *gag*-transcription unit present in pONY2.13LacZ.

Figure 5 is a representation of vectors derived from the EIAV genome.

Figure 6 is a representation of gagpol constructs derived from EIAV.

Figure 7 is a representation of an EIAV vector comprising an *S2* deletion

Figure 8 is a representation of EIAV *gagpol* constructs having deleted *S2* and *dUTPase* genes.

Figure 9 is a representation of an EIAV minimal vector.

Figure 10 illustrates a gel showing an analysis of EIAV *gagpol* constructs according to the invention.

Figure 11 shows examples of the pONY4 vectors.

Figure 12 shows two SIN vectors.

Figure 13 is a representation of a vector with a split polyA signal.

Figure 14 is a representation of a vector with a split polyA signal.

Figure 15 is a representation of a vector with a split polyA signal.

Figure 16 shows construction of pONY4-GFP with a split polyA signal.

Figurer 17 shows construction of a MLV/EIAV vector.

Figure 18 shows primers for construction of MLV/EIAV vectors.

Figure 19 shows complete sequence of pONY-mouse.

Figures 20 and 21 give PCR priming.

Figure 22 shows pEMVA4 (after PCR with primers EMVA 1-8).

Figure 23 shows pEMVA4.

Figure 24 shows pEMVA5.

Figures 25 and 26 show an example of hammer-head strategy for 5' end formation.

Figure 27 shows pEMVA6.

Figure 28 shows pEMVA7 and pSyn pONY4.1.

Figure 29 shows EMVA 10/11.

Figure 30 shows pEMVA9.

Figure 31 shows pEMVA10.

Figure 32 shows pLWHORSE3.1.

Figure 33 shows pMCRev.

Figure 34 shows pYFVSVG.

Figure 35 shows pYFAmpho.

Figure 36 shows recombinant MVA constructs.

Figure 37 shows the complete sequence of pSC65.

Figure 38 shows the complete sequence of pLW22

In more detail, FIGURE 1. Plasmids used in this study. The genomic organization of EIAV is indicated including splice donor (d1, d2 and d3) and splice acceptor sites (a1, a2 and a3). The positions of *gag, pol, env, tat, rev, S2* and the viral LTRs are also shown. Plasmid pESP is an EIAV vector genome containing the SV40 promoter and the puromycin resistance gene. Plasmid pONY3 is an EIAV gagpol expression plasmid. pONY2.1nlslacZ is an EIAV vector genome containing a HCMV IE enhancer/promoter and a β-galactosidase gene (nlslacZ).

Figure 2 shows PCR analysis of integrated EIAV vector. PCR was performed with either genomic DNA from EIAV vector transduced cells (lanes 1 and 5) or mock transduced cells (lanes 2 and 6). pONY2.1nlsLacZ (lanes 3 and 7) and pONY3 (lanes 4 and 8) were used as controls. A. PCR detection of EIAV LTRs. B. PCR detection of pol.

TABLE 2. Transduction of dividing and non-dividing cells. 293T cells were treated with (open columns) or without (shaded columns) aphidicolin according to the method of Lewis et al. (Lewis, P. F., and M. Emerman. 1994. J Virol. 68:510-6.) and then transduced with either EIAV vector PONY2.1nlsLacZ or MLV vector HIT111 (Soneoka et al 1995 Nucl. Acids Res. 23:628-633). 48 hours later the cells were stained with X-gal. Titers were averaged from two independent experiments and calculated as lac Z colony forming units per ml. There was no more than 10% variation between experiments.

Figure 10 shows analysis of gagpol expression constructs. 30μg of total cellular protein was separated by SDS/Page electrophoresis, transferred to nitro-cellulose and probed with anti-EIAV antibodies. The secondary antibody was anti-Horse HRP (Sigma). Titres were averaged from three independent experiments and calulated as lacZ forming units per ml. There was no more than 10% variation between experiments. pONY2.1nlslacZ and the envelope expression plasmids were co-transfected with the EIAV *gagpol*.

Example 1 – Construction of EIAV vectors containing deleted gag genes.

In order to construct a replication incompetent EIAV vector system we have used, as a starting point, an infectious proviral clone pSPEIAV19 (accession number: U01866), described by Payne et al. (1994, J Gen Virol. 75:425-9). An initial EIAV based vector was constructed by simply deleting part of *env* by removing a *Hind III/Hind III* fragment corresponding to coordinates 5835/6571 according to the numbering system of Payne et al. (ibid.). This fragment was replaced with the puromycin resistance gene under the control of the SV40 early promoter from pTIN500 (Cannon et al 1996 J. Virol. 70:8234-8240) to create pESP (Figure 1). Viral stocks were produced by calcium phosphate transfection of 293T cells (Soneoka et al 1995 Nucl. Acids Res. 23:628-633) with pESP and pRV67 (Kim et al 1998 J. Virol. 72(1):811-6) a plasmid in which the vesicular stomatitis virus glycoprotein (VSV-G) is expressed from the HCMV-IE enhancer/promoter. Alternatively other VSV-G expression plasmids can be used eg Yee, et al 1994 PNAS 91:9564-9568. The resulting supernatant was used to transduce human

kidney (293T) and canine osteosarcoma cells (D17) as follows. 48 hours post-transfection tissue culture fluid was collected and filtered through 0.45μm filters. Ten-fold dilutions were made in culture medium containing polybrene at 8μg/ml and then 500μl aliquots placed on D17 cells seeded at 1.6 x 105/well in 12 well plates on the previous day. Two hours later 1 ml of culture media was added. Two days later puromycin was added to a final concentration of 4ug/ml and incubation was continued for a further 7 days. As a positive control, a Murine leukaemia virus (MLV) based vector (pTIN500) containing the puromycin resistance gene under the control of the SV40 early promoter was used in conjunction with pHIT60 (MLV gagpol) and pRV67 (Cannon et al 1996 J. Virol. 70:8234-8240). No resistance colonies were detected on either cell type after 7 days of puromycin selection with the EIAV vector. The MLV vector produced 5.0 x 10^4 c.f.u./ml on 293T cells and 1.0 x 10^4 c.f.u./ml on D17 cells.

The likely explanation for this result is that the EIAV LTR is not functional in human cells in the absence of tat and so insufficient amounts of the critical components such as gag-pol and tat are produced.

A further vector system was therefore constructed comprising three transcription units to produce the following: 1) vector genome RNA; 2) env and 3) gag-pol. In order to ensure that sufficient of each component is produced, the env and gag-pol transcription units are transcribed from a promoter-enhancer active in the chosen human packaging cell line. In this way, sufficient gag-pol and, most likely tat, are produced to ensure efficient production of transduction-competent vector particles.

The vector genome was constructed which has the reporter gene within the pol region of the genome as follows. The plasmid designated pONY1 was constructed by inserting the EIAV LTR, amplified by PCR from pSPEIAV19, into pBluescript II KS+ (Stratagene). The 5' LTR of EIAV clone pSPEIAV19 was PCR amplified using pfu polymerase with the following primers:

5' GCATGGACCTGTGGGGTTTTTATGAGG

3' GCATGAGCTCTGTAGGATCTCGAACAGAC

The amplicon was blunt ended by 5' overhang fill-in and inserted into pBluescript II KS+ cut with *Bss HII* which had been blunt ended by 3' overhand removal using T4 DNA polymerase. This construct was called pONY1 and the orientation was 5' to 3' in relation to β-galactosidase of pBluescript II KS+. Sequencing of pONY1 revealed no mutations.

Vector genome pSPEIAV19DH was cut with Mlu I (216/8124) and inserted into pONY1 Mlu I cut (216) to make pONY2. A *Bss HII* digest (619/792) of pBluescript II KS+ was carried out to obtain the multiple cloning site. This was blunt ended by 5' overhang fill-in and ligated to pONY2 cut with *Bgl II* and *Nco I* (1901/4949) and blunt ended by 5' overhang fill-in. The orientation was 3' to 5' in relation to the EIAV sequence. This was called pONY2.1. pSPCMV was created by inserting pLNCX (Accession number: M28246) (*Pst I/Hind III*) into pSP72 (Promega). The β-galactosidase gene was inserted from pTIN414 (Cannon PM et al J. Virol. 70, 8234-8240) into pSP72 (*Xho I/Sph I*) to make pSPlacZ. The 5' end to the β-galactosidase gene was replaced by the SV40 T antigen nuclear localization signal from pAD.RSVbgal (J. Clin. Invet. 90:626-630, 1992). pAD.RSVbgal was cut with *Xho I/ Cla I* and inserted into *Xho I/ Cla I* pSPlacZ to make pSPnlslacZ. The CMV nuclear localizing and non nuclear localizing β-galactosidase from pSPlacZ and pSPnlslacZ was cut out with Pst I and inserted into the Pst I site of pONY2.1 in the 5' to 3' orientation of EIAV. These were called pONY2.1nlslacZ and pONY2.1lacZ.

An EIAV gagpol expression plasmid (pONY3) was then made by inserting Mlu I/Mlu I fragment from pONY2ΔH into the mammalian expression plasmid pCl-neo (Promega) such that the gag-pol gene is expressed from the hCMV-MIE promoter-enhancer. In particular, gagpol pSPEIAV19DH was cut with Mlu I (216/8124) and inserted into pCI-Neo(Promega) Mlu I cut (216) to make pONY3. Plasmid pONY3 should not produce a functional genome because it lacks the appropriate LTR sequences. Virus was produced by transient three plasmid cotransfection of 293T cells with pRV67, pONY3 and pONY2.10nlsLacZ as described for MLV-based vectors (Soneoka et al 1995 Nucl. Acids Res. 23:628-633) and then used to transduce 293T cells and D17 cells as follows. 48

hours post-transfection tissue culture fluid was collected and filtered through 0.45μm filters. Ten-fold dilutions were made in culture medium containing polybrene at 8μg/ml and then 500ul aliquots placed on D17 cells seeded at 1.6 x 105/well in 12 well plates on the previous day. Two hours later 1ml of culture media was added and incubation continued for 48 hours prior to assessment of LacZ gene expression using the X-gal staining procedure. for E coli β-galactosidase (macGregor et al 1991 Methods in Molecular Biology Vol 7 ed EJ Murray p217-235). In both cases the virus transduced the cells at frequencies of about 10^5 LacZ-transducing cell - forming - unit (i.f.u.)/ml which was about 10-fold less than with the MLV-based vector produced from pHIT111. These data showed that we had produced an EIAV-based vector system and also suggested that replacement of the *Hind III/Hind III* fragment in *env* with foreign DNA may disrupt the function of the genome.

We next characterized the ability of the EIAV vector particles to be pseudotyped with envelope proteins from other viruses. pONY2.10nlsLacZ and pONY3 were cotranfected with envelope expression plasmids producing MLV amphotropic (pHIT456) and MLV ecotropic (pHIT123) envelopes (Soneoka et al 1995 Nucl. Acids Res. 23:628-633) as well as VSV-G (pRV67) (Table 1). pHIT111 (MLV vector genome) and pHIT60 (MLV gagpol expression plasmid) were cotransfected with the envelope plasmids as positive controls (Table 1). The viral supernatants were used to transduce a variety of cell lines including human kidney (293T), murine embryo (NIH3T3) and canine osteosarcoma (D17). As expected, the cell tropism of the virus was largely determined by the envelope. EIAV could be pseudotyped with amphotropic envelope, but transduction efficiencies varied. The amphotropic pseudotyped virus gave titres of about 102 on D17 cells, 103 on NIH3T3 cells and 104 on 293T cells. The reason for these differences was not pursued. EIAV could also be pseudotyped with the MLV ecotropic envelope and these viruses transduced NIH3T3 cells at titres of 104 l.f.u./ml. EIAV, pseudotyped with VSV-G envelope, transduced all the cell lines tested. The titer varied between the different envelopes and cell types but overall efficiencies were relatively high for the non-murine cells, but still lower than with a murine vector system. Taken together, these data show

that the EIAV vector system is not dependent on the EIAV envelope and can be effectively pseudotyped with three envelopes conferring broad host range. This makes this system as generally useful as current MLV-based systems.

EIAV vectors can also be pseudotyped in the same manner using the RD114 envelope, for instance using pRDF (Cosset et al 1995 J. Virol. 69: 7430-7436).

In order to characterize the transduction events further we carried out a PCR analysis of 293T cells transduced by the EIAV vector pseudotyped with VSV-G. In particular we asked if the vector genome, as opposed to a recombinant with the gagpol expression plasmid, pONY3, had been the transduction vehicle for the β-galactosidase gene. PCR amplification using primers specific for the EIAV LTR gave the expected PCR product of 310bp when genomic DNA isolated from transduced cells was used (Figure 2A, lane 1). No PCR product was detected when mock transduced 293T cell DNA was used as template (Figure 2A, lane 2). pONY2.10nlsLacZ was used as a positive control (Figure 2A, lane 3). No PCR product was detected when pONY3 was used as a template (Figure 2A, lane 4). The lack of a PCR product, when using pol specific primers, (Figure 2B) confirmed that no gagpol sequences from pONY3 had integrated into the host chromosomes. Taken together these data show that the authentic vector genome had transduced the cells.

In order to determine if the EIAV vector retained the ability to transduce non-dividing cells, 293T cells were arrested in G1/S phase by treatment with aphidicolin according to published procedures (Lewis and Emerman 1994) and then transduced with EIAV-based and MLV-based vectors pseudotyped with VSV-G. The transduction efficiency of the MLV vector was lower by four orders of magnitude in aphidicolin treated cells as compared to untreated cells. The incomplete block to cell transduction by MLV was probably due to a small population of dividing cells. In contrast, no significant difference was observed in the case of the EIAV-based vector. This demonstrates that the EIAV vector, like HIV vectors, can efficiently transduce non-dividing cells.

The vector genome pONY2.10lacZ contains 1377nt of *gag*. RNA secondary structure prediction ("http://www.ibc.wustl.edu/~zuker/rna/") was used to identify possible stem-loop structures within the leader and the 5' end of gag. Based on these predictions four deletions were made within the gag region of pONY2.10lacZ (figure 1). Deletions were made by PCR mutagenesis using standard techniques.

pONY2.11lacZ contains 1377nt of *gag* (deleted from position 1901nt)
pONY2.111acZ contains 354nt of *gag* (deleted from position 878nt)
pONY2.12lacZ contains 184nt of *gag* (deleted from position 708nt)
pONY2.13lacZ contains 109nt of *gag* (deleted from position 633nt)
pONY2.14lacZ contains 2nt of *gag* (deleted from position 526nt)

These vectors were used in a three plasmid cotransfection as described for MLV-based vectors (Soneoka et al 1995 Nucl. Acids Res. 23:628-633) and the virus generated was titred on 293T and D17 cells.

It was found that the first 109nt of *gag* coding sequence were needed for maximal packaging in addition to the un-translated region; pONY2.13lacz (Table 2). Similar titres were found on D17 cells. The predicted secondary structure of the gag sequence derived RNA in pONY2.13lacZ is shown in Figure 4.

Based on the secondary structure prediction in Figure 4, four further deletions were made within the area upstream and downstream of the major splice donor codon in pONY2.13lacZ.

pONY2.21lacZ contains deleted between position 409 to 421nt
pONY2.22lacZ contains deleted between position 424 to 463nt
pONY2.23lacZ contains deleted between position 470 to 524nt
pONY2.24lacZ contains deleted between position 529 to 582nt

pONY2.25lacZ contains deleted between position 584 to 645nt
pONY2.26lacZ contains deleted between position 409 to 421nt and between position 470 to 542nt.

These vectors were used in a three plasmid co-transfection as described above and the virus generated was titred on D17 cells. It was found that deletions within this region severely affected the titre of the virus (Table 3). Constructs pONY2.23 and 2.26 gave the lowest titre. These both contained the deletion between position 470 to 524nt. The least severe deletion was the one between position 409 to 421nt. Based on this information the region around the major splice donor is useful for optimal packaging.

Similar secondary structure predictions and deletion analysis may be used to identify the packaging signal in other non-primate lentiviruses.

TABLE 1. Transduction efficiency of viral vectors.

Vector	Envelope	Titer (l.f.u./ml)[a]		
		D17	NIH3T3	293T
pONY2.1nlslacZ	Mock	< 1	< 1	< 1
pONY2.1nlslacZ	pHIT456 (MLVamp)	1.0×10^2	8.4×10^2	2.0×10^4
pONY2.1nlslacZ	pHIT123 (MLVeco)	< 1	1.5×10^4	< 1
pONY2.1nlslacZ	pRV67 (VSVG)	1.0×10^5	3.6×10^3	2.0×10^5
pHIT111	Mock	< 1	< 1	< 1
pHIT111	pHIT456 (MLVamp)	1.3×10^5	2.6×10^6	2.0×10^7
pHIT111	pHIT123 (MLVeco)	< 1	2.8×10^6	< 1
pHIT111	pRV67 (VSVG)	3.0×10^6	2.0×10^5	5.0×10^6

[a] Each cell type was transduced and stained for β-galactosidase activity 48 hours after transduction of the target cells. Titers were averaged from three independent experiments and calculated as lac Z forming units per ml. There was no more than 10% variation between experiments. pONY2.1nlsLacZ and the envelope expression plasmids were contransfected with the EIAV *gagpol* expression plasmid (pONY3). pHIT111 and the envelope expression plasmids were cotransfected with the MLV *gagpol* expression plasmid (pHIT60).

Table 2

Vector Genome	Titre (I.f.u/ml)
PONY2.10	3.30E+04
PONY2.11	1.60E+05
PONY2.12	1.40E+05
PONY2.13	1.70E+05
PONY2.14	5.40E+02
Mock	1.0E+01

Table 3

Vector Genome	Titre (I.f.u/ml)
2.21	1.20E+04
2.22	3.80E+03
2.23	1.20E+02
2.24	5.20E+02
2.25	5.60E+02
2.26	1.00E+02
2.13	4.00E+04

Example 2 – Construction of pEGASUS-1

An EIAV – based vector was made (pEGASUS-1) that contains only 759nt of EIAV sequences (268nt-675nt and 7942nt-8292nt) as follows.

Sequences encompassing the EIAV polypurine tract (PPT) and the 3'LTR were obtained by PCR amplification from pONY2.10LacZ using primers PPTEIAV+ (Y8198): GACTACGACTAGTGTATGTTTAGAAAAACAAGG, and 3'NEGSpeI (Y8199):CTAGGCTACTAGTACTGTAGGATCTCGAACAG. The product was purified, digested with *SpeI* (ACTAGT) and ligated into pBS II KS+ which had been prepared by digestion with *SpeI* and treatment with alkaline phosphatase. Colonies obtained following transformation into E. coli, XL-1Blue were screened for the presence of the 3'LTR in the orientation in which the U5 region of the 3'LTR was proximal to the *NotI* site of the pBS II KS+ linker. The sequence of the cloned insert was determined and showed that it contained only one change from the EIAV clone pSPEIAV19 (AC: U01866). This was a 'C' insertion between bases 3 and 4 of the R region. The same change was found in the template used in the PCR reaction. The clone was termed pBS.3'LTR.

Next the reporter gene cassette, CMV promoter/LacZ, was introduced into the *PstI* site of pBS.3'LTR. The CMV/LacZ cassette was obtained as a *PstI* fragment from pONY2.10LacZ (see above). The ligation reaction to join the above fragments was transformed into E. coli, XL-1Blue. A number of clones in which the CMV/LacZ insert was orientated so that the LacZ gene was proximal to the 3'LTR were assessed for activity of the CMV/LacZ cassette by transfection into the cell line 293T using standard procedures. A clone which gave blue cells at 48 hours post-transfection following development with X-gal was selected for further use and termed pBS CMVLacZ.3'LTR.

The 5'region of the EIAV vector was constructed in the expression vector pCIEneo which is a derivative of pCIneo (Promega) modified by the inclusion of approximately 400 base pairs derived from the 5'end of the full CMV promoter as defined previously. This 400 base pair fragment was obtained by PCR amplifcation using primers VSAT1 (GGGCTATATGAGATCTTGAATAATAAAATGTGT) and VSAT2 (TATTAATAACTAGT) and pHIT60 as template. The product was digested with *BglII* and *SpeI* and ligated into pCIneo which had been digested similarly.

A fragment of the EIAV genome running from the R region to nt 150 of the *gag* coding region (nt 268 to 675) was amplified with primers CMV5'EIAV2 (Z0591)(GCTACGCAGAGCTCGTTTAGTGAACCGGGCACTCAGATTCTG: and 3'PSI.NEG (GCTGAGCTCTAGAGTCCTTTTCTTTTACAAAGTTGG) using as template DNA. The 5'region of the primer CMV5'EIAV2 contains the sequences immediately upstream of the CMV promoter transcriptional start site and can be cut with *SacI*. 3'PSI.NEG binds 3' of the EIAV packaging sequences as defined by deletion analysis (above) and contains an *XbaI* site. The PCR product was trimmed with *SacI* and *XbaI* and ligated into pCIEneo which had been prepared for ligation by digestion with the same enzymes. This manipulation places the start of the EIAV R region at the transcriptional start point of the CMV promoter and transcripts produced thus start at the genuine start position used by EIAV and extend to the 3'-side of the packaging signal. Clones which appeared to be correct as assessed by restriction analysis were sequenced. A clone termed pCIEneo.5'EIAV was selected for further work.

In the next step the CMVLacZ and 3'LTR cassette in pBS.CMVLacZ.3'LTR was introduced into pCIEneo.5'EIAV. pBS.CMVLacZ.3'LTR was digested with *ApaI*, the 3'overhangs removed with T4 DNA polymerase, then digested with *NotI*. The fragment containing the CMVLacZ.3'LTR was purified by standard molecular biology techniques. The vector for ligation with this fragment was prepared from pCIEneo.5'EIAV by digestion with *SalI*, followed by filling-in of the 5'overhangs using T4 DNA polymerase. The DNA was then digested with *NotI* and purified prior to use in ligation reactions.

Following transformation into E.coli, XL-1Blue colonies were screened for the presence of the insert by restriction analysis to identify the required clone, designated pEGASUS-1.

The function of the pEGASUS-1 EIAV vector was compared to pONY2.10LacZ using the three plasmid co-transfection system as described in Example 1. Comparable titres were obtained from both vectors indicating that pEGASUS-1 contains all the sequences required for packaging with good efficiency.

Example 3- Introduction of RRE's into EIAV vectors

Further improvements to the EIAV vector pEGASUS-1 may be made by introduction of additional elements to improve titre. A convenient site for the introduction of such elements is the SalI site which lies between the *XbaI* to the 3' of the packaging signal and upstream of the CMV/LacZ cassette of pEGASUS-1. For example the RRE from HIV or EIAV can be inserted at this site.

The HIV-1 RRE was obtained from the HIV-1 molecular clone pWI3 (Kimpton and Emerman 1992 (J. Virol. 66: 2232-2239) by PCR amplification using primers RRE(+) GTCGCTGAGGTCGACAAGGCAAAGAGAAGAG and RRE(-) GACCGGTACCGTCGACAAGGCACAGCAGTGG. The fragment of DNA and pEGASUS-1 were digested with *SalI* and following ligation, transformed into E.coli, XL-1 Blue. Colonies were screened for the presence of the HIV RRE and two clones, with the HIV RRE in either the positive or negative orientation, used for further work These vectors, pEGASUS-2.HIV RRE(+) or pEGASUS-2.HIV RRE(-) can be tested in 293T cells by carrying out a four plasmid co-transfection in which the plasmid pCIneoHIVrev, expressing the rev protein from HIV-1 is co-transfected with vector, pONY3 and pRV67 plasmids

The EIAV RRE as defined previously (Martarano et al 1994) was obtained by PCR amplification as follows. Using pONY2.10LacZ as template 2 amplifications were

performed to obtain the two parts of the EIAV RRE. The 5'-element was obtained using primers ERRE1 (TTCTGTCGACGAATCCCAGGGGGAATCTCAAC) and ERRE2 (GTCACCTTCCAGAGGGCCCTGGCTAAGCATAACAG) and the 3'element with ERRE3 (CTGTTATGCTTAGCCAGGGCCCTCTGGAAGGTGAC) and ERRE4 (AATTGCTGACCCCCAAAATAGCCATAAG). These products will anneal to each other hence can be used in second PCR reaction to obtain a DNA which 'encodes' the EIAV RRE. The PCR amplification is set up with out primers ERRE1 and ERRE4 for the first 10 cycles and then these primers are added to the reaction and a further 10 cycles of amplification carried out. The resulting PCR product and pEGASUS-1 were digested with *SalI*, ligated and transformed into E.coli XL-1Blue. Clones in which the EIAV RRE was in either the positive or negative orientations were selected for further work. The activity of these vectors was assessed in 4-way co-transfectioand pEGASUS-1 were digested with *SalI*, ligated and transformed into E.coli XL-1Blue. Clones in which the EIAV RRE was in either the positive or negative orientations were selected for further work. The activity of these vectors was assessed in three palsmid co-transfections, (EIAV rev being supplied by pONY3) or in 4-plasmid co-transfection experiments as described above, but using pCIneo.EIAV Rev to supply additional EIAV rev.

For construction of pCIneo EIAV REV the EIAV REV encoding sequences were derived by PCR amplification. The EIAV REV sequences were obtained using a two step'overlapping' PCR amplification procedure as described above for the EIAV RRE. Template for the two reactions was pONY3 and primers for the 5'fragment were EIAV REV5'O (CCATGCACGTCTGCAGCCAGCATGGCAGAATCGAAG) and EAIV REV IN (CCTGAGGATCTATTTTCCACCAGTCATTTC) and for the 3'product EIAV REV IP (GTGGAAAATAGATCCTCAGGGCCCTCTGG) and EIAV.REV3'O (GCAGTGCCGGATCCTCATAAATGTTTCCTCCTTCG). The second PCR amplification was carried out with primers EIAV REV5'O and EIAV REV3'O being added after 10 cycles. The resulting product was ligated with the PCR fragment 'TA' cloning vector pCR2.1 (Invitrogen) the orientation of the EIAV REV insert was assessed by restriction enzyme analysis and the presence of the correct EIAV REV sequence

confirmed. The construct was called pTopoRevpos. The EIAV REV insert was excised from pTopoRevpos by digestion with *SpeI* and *NotI* and ligated into pCIneo which had been digested with *NheI* and *NotI*.

Example 4 – Transduction of human macrophages

Primary human monocytes were obtained from leukocyte–enriched blood (from the National Blood Transfusion Service, Southmead Rd Bristol, UK) as follows. Peripheral blood mononuclear cells (PBMC) were enriched by centrifugation above a Ficoll discontinuous gradient (Pharmacia) according to the manufacturer's instructions. Macrophages were obtained from this cell population by adherence to tissue culture plastic over 7 days in RPMI 1640 medium (Dutch modified ; Sigma) containing 2% heat-inactivated human AB serum (Sigma) or 10% FCS (Sigma). Non-adherent cells were removed by extensive washing of the plates with medium.

Virus for transduction experiments was obtained by three plasmid co-transfection into 293T cells. The vector for the experiments was a pONY2.13 derivative in which the CMV/LacZ reporter cassette had been replaced with CMV/green fluorescent protein (GFP).

Vector pONY2.13GFP was made as follows. The sequence encoding the red-shifted GFP and eukaryotic translation signals was cut out of pEGFP-N1 (Clontech "http://www.clontech.com/") with *BglII* and *XbaI* and ligated into the general cloning vector pSP72 (Promega) which had been prepared by digestion with the same enzymes. The GFP-encoding sequences were then excised using *XhoI* and ligated into pONY2.13 which had been cut with *XhoI* (thereby releasing the LacZ coding region). Following transformation into E.coli, XL-1Blue clones in which the orientation of the GFP insert with respect to the CMV promoter was such that expression would be expected were determined restriction analysis and expression of GFP confirmed by transfection of DNA into 293T cells.

Vector was recovered by three plasmid co-transfection into 293T cells and harvested at 42-48 hours post-transfection: tissue culture fluid was 0.45(m-filtered and virus was then pelleted by centrifugation at 50,000g (20Krpm), for 90 minutes at 4(C in a SW40Ti rotor. Virus was resupended in 50-100(l of complete media for 2 hours and then used in transduction experiments. Transductions with pONY2.13GFP vector were carried out as follows. Macrophages , seeded at 5 x 105 per well of 48-well plates were washed once with medium and then 300(l of medium was put back on the cells. Virus was added to the medium and gently pipetted up 2-3 times to ensure mixing. Transduction efficiency was assessed at 3-5 days post-transduction. The number of transduced macrophages was determined using a fluorescence microscope. Expression of GFP can be monitored for extended periods, e.g., up to several weeks. Alternatively, transductions can be carried out with vectors carrying the LacZ marker. In such experiments the transduction frequency is assessed by detecting the presence of β-galactosidase using immunological procedures.

Example 5 – Introduction of EIAV vectors in vivo in rat brain

Adult Wistar rats were anaesthetised with a solution containing 1 part Nembutal (0.1ml/35gm body weight) 1 part Novetal (.1ml/35 gm body weight) and 2 parts dH2O, and placed into a stereotaxic apparatus. A midline incision was made along the rostral-to-caudal length of the scalp and the skin deflected back to expose the skull. Using stereotaxic coordinates (measured from Bregma) of 3.00mm posterior and 3.00 lateral, a 1mm diameter hole was drilled into the skull. Unilateral intracortical injections of EIAV vectors were then made using a 10μl Hamilton syringe or a 1.0μl fine glass capillary to various depths from the surface of the brain. The syringe was left in place an additional 5 min to prevent reflux. Control animals receive a single 10μl intracortical injection of saline with the Hamilton or 1.0μl with the fine glass capillary. Animals were then sutured and left to recover. Forty-eight hours later, these animals were deeply anesthetized as described above and perfused through the heart with 200ml of phosphate-buffered saline (PBS). The brains were then dissected out, frozen into dry-ice cooled isopentane (-30oC)

and cut coronally at 10μm with a cryostat. Every 5th section through the injection site and 2mm rostral and caudal are collected onto Super-Frost slides, fixed and either X-gal or immunostained or stained with Cresyl Violet.

Example 6 – Transduction of Bronchial Cells differentiated in Culture

Epithelial cells can be differentiated to form epithelia-like monolayers which display (>1000 Ω cm^3) electrical resistance and a cuboidal morphology. There are various wasys to do this for example Fuller et al 1984.This creates polarized cells. This polarity is functional and mimics epithelial cells *in vivo*. Thus EIAV vectors can be used to transduce these cells either through the basolateral surface or the apical surface using vectors and preparations as described in Examples 1 –3.

Example 7 - A Minimal EIAV System

In order to eliminate the risk of accessory genes or coding sequences having deleterious effects in therapeutic applications, vector systems lacking *tat*, *S2* and the *dUTPase* are constructed.

Construction of S2 Mutants

A) Vector Genome

pONY2.13lacZ contains 109nt of *gag* (deleted from nucleotide positions 633 to 4949) (pONY2.13lacZ is described above). This vector is used to make an EIAV vector genome from which S2 expression is eliminated by deletion from nucleotide positions 5345 to 5397. This removes the ATG start codon of *S2* and the start codon of *env*. To make the deletion within *S2*, PCR is carried out with SY2/SY5 and SY3/SY4 using pONY2.13 DNA as template. The two PCR products are pooled and PCR is carried out with primers SY5 and SY3. The 1.1kb product is ligated into pGEMT-easy (Promega) to make pGEMS2 and sequenced to confirm the deletion. pONY2.13lacZDS2 is made by cutting out the 1.1kb S2 region from pGEMS2 with *Cel* II and ligating it into pONY2.13lacZ.

B) Gagpol Construct

The same region of S2 is deleted in pONY3 to prevent recombination between pONY3DS2 and pONY2.13DS2 reconstituting the *S2* gene. pONY3DS2 is made by PCR amplification with SY1/SY2 and SY3/SY4 using pONY3 DNA as template. The two PCR products are pooled and PCR is carried out with primers SY1 and SY3. The 0.7kb product is ligated into pGEMT-easy (Promega) to make pGEMS22 and sequenced to confirm the deletion. The 0.7kb S2 coding region is excised of pGEMS22 with *Not* I and inserted into pBluescript KS+ (Stratagene) to make pBPCRS2. The *Eco* RV and *Nco* I fragment from pONY3 (2.2kb) is inserted into pBPCRS2 cut with *Eco* RV and *Nco* I to make pBpONYS2. This is then cut with *Eco* RV and *Cel* II (2.9kb fragment) and inserted into pONY3 cut with *Eco* RV and *Cel* II to thereby making pONY3DS2.

Construction of dUTPase Mutant

pONY3DdUTPase is made by site directed mutagenesis of nucleotide 4176 from a T to an A residue (Payne *et al.*, Virology, 210:302-313). This mutates the aspartic acid to a glutamic acid. This is done by PCR amplification using PCR primers dUTPaseF and dUTPaseR. The template DNA is pONY3. The PCR product is inserted into pGEMT-easy and sequenced to confirm the mutation. This is called pGDdUTPase. pONY3 is cut with *Not* I and *Eco* RV (4.6kb) and inserted into pBluescript KS+ (Stratagene) to make pBEV. The pGDdUTase is cut with *Pac* I and *Pst* I and the 0.4kb band inserted into pBEV cut with *Pac* I and *Pst* I. This is called pONY3pBDUTPase. This is then inserted into pONY3 via *Not* I and *Eco* RV (4.6kb) to make pONY3DdUTPase.

Construction of the S2 and dUTPase Double Mutant

To make the double mutant of pONY3 the construct pBpONYS2 is used. pGDdUTPase is cut with *Pac* I and *Pst* I and the fragment inserted into pBpONYS2 cut with *Pac* I and *Pst* I to make construct pS2DdUTPase. This is then cut with *Eco* RV and *Cel* II and inserted into pONY3 cut with *Eco* RV and *Cel* II to make pONY3DS2DdUTPase.

Analysis of S2 and dUTPase mutants

pONY2.13lacZDS2, pONY3DdUTPase and pONY3DS2DdUTPase vectors are used in a number of combinations in three plasmid co-transfections to generate virus as described for MLV-based vectors (Soneoka *et al* 1995 Nucl. Acids Res. 23:628-633) and the virus generated is titred on 293T and D17 cells, in either dividing or non-dividing states. Cells are arrested in G_1/S phase by treatment with aphidicolin (9) and then transduced with EIAV-based and MLV-based vectors pseudotyped with VSV-G (Table 4). The transduction efficiency of the MLV vector is lower by four orders of magnitude in aphidicolin treated cells as compared to untreated cells. The incomplete block to cell transduction by MLV is probably due to a small population of dividing cells. In contrast, no significant difference is observed in the case of the EIAV-based vectors. This demonstrates that the EIAV-based system does not require *S2* or *dUTPase* either for production or transduction. Payne *et al.*, (Payne *et al.*, Virology, 210:302-313) and others have shown that EIAV *dUTPase* is required for the infection of horse macrophages. This may represent a restriction in infection of macrophages by EIAV.

The properties of the *S2* and *dUTPase* mutants are tested by transduction of hippocampal embryonic day 14 neuronal cells cultured in minimal media for 7 days. No significant difference is found between the various EIAV vectors. However a much reduced transduction efficiency is seen for the MLV vector. This indicates that *S2* and *dUTPase* is not required for the transduction of physiologically non-dividing cells.

In summary we can conclude that *tat*, *S2* and *dUTPase* are not required in any part of the vector system for vector production or transduction.

Example 8 - Addition of Rev/RRE

The construction of pEGASUS-1 has been described above. This vector contains 759bp of EIAV sequence. The introduction of the EIAV RRE (0.7kb) into pEGASUS-1 to

produce pEGASUS/RRE resulted in a four-fold increase in the titre when Rev is provided in trans (Table 2). This vector now contains 1.47kb of EIAV.

Example 9 - Construction of improved gagpol expression plasmids

In pONY3 there is an extended 5' untranslated region before the start of the *gagpol* coding sequence. It is likely that this unusually long sequence would compromise expression of the *gagpol* cassette. To improve *gagpol* expression pONY3 is modified to remove the remaining 5' LTR. This is done by cutting pONY3 with *Nar* I and *Eco* RV. The 2.4kb fragment is inserted into pBluescript KS+ (Stratagene) at *Cla* I and *Eco* RV sites to make construct pBSpONY3.0. pBSpONY3.0 is cut with *Xho* I and *Eco* RV. The 2.4kb fragment is inserted into pONY3 at *Xho* I and *Eco* RV to make pONY3.1.

This manipulation removes the 5' LTR up to the *Nar* I site within the primer binding region (386nt). This construct gives a two fold increase in titre and increased protein expression (Figure 10).

pONY3.1 like pONY3 encodes *gag*, *gagpol*, *Tat*, *S2* and *Rev*. Since the *S2* mutation experiments showed that S2 is not required either in the production system or in the EIAV vector genome it is possible to design a *gagpol* expression constructs without S2. Two such constructs, pHORSE and pHORSE3.1, are produced.

pHORSE is made by PCR amplification with EGAGP5'OUTER/EGAGPINNER3 and EGAGP3'OUTER/EGAGPINNER5 using pONY3 as template DNA. The two PCR products are purified pooled and re-amplified using primers EGAGP5'OUTER /EGAGP3'OUTER. This product is inserted into the *Xho* I and *Sal* I sites of pSP72 to make pSP72EIAVgagpolO'lap. pONY 3 is cut with *Pvu* II and *Nco* I and the 4.3kb fragment is inserted into pSP72EIAVgagpolO'lap cut with *Pvu* II and *Nco* I to make pSPEGP. This is cut with *Xho* I and *Sal* I (4.7kb) and inserted into pCI-Neo at the *Xho* I

and *Sal* I sites. This construct is called pCIEGP. The RRE is cut out from pEGASUS with *Sal I* (0.7kb) and inserted into pCIEGP construct at the *Sal* I site to make pHORSE.

When this construct is assayed for protein expression in the presence or absence of pCI-Rev (a construct expressing the EIAV Rev open reading frame, see above) it is found to be Rev dependent as expected. However, protein expression is much lower than from pONY3.1. In addition when used in virus production the titre is found to be 100 fold lower than that from pONY3.1.

Unexpectedly when the leader sequence (comprising sequences from the end of U5 of the 5' LTR to the ATG start of *gag* 383 – 524nt) of pONY3.1 is inserted into pHORSE, to make pHORSE3.1, protein expression and virus production improved. pHORSE3.1 is made by replacing the 1.5kb *Xho* I/*Xba* I of pHORSE with the1.6kb *Xho* I/*Xba* I of pONY3.1. Titres obtained with pHORSE3.1 are similar to that of pONY3.1. The reason for the slightly lower titre of pHORSE3.1 compared to pONY3.1 may be due the requirement for a four plasmid co-transfection with pHORSE3.1 (due to the Rev dependence of this system). We can conclude therefore that a minimal EIAV vector system should have this leader for maximum *gagpol* expression.

When pHORSE3.1, pRV67, pCIRev and pEGASUS/RRE are used in a four plasmid co-transfection (Table 6) virus is produced at a high titre (2.0×10^4 l.f.u./ml). This system lacks the second exon of *Tat* which is responsible for *Tat* transactivation (Southgate *et al.*, J. Virology, 1995, 69:2605-2610). This demonstrates that the *Tat* is not required for the EIAV-based vector system.

By engineering the backbone of pHORSE3.1 to express Rev (replacing the Neo open reading frame with that of EIAV Rev) the requirement of a four plasmid co-transfection was eliminated. This was done by cutting pCI-Neo with *Stu* I and *Bst* XI and filling in the 5' overhangs with T4 DNA polymerase. This produced a vector fragment of 4.6kb into which the Rev open reading frame from pTopoRevpos (cut with *Sac* I and *Xba* I giving a

0.6kb band in which the 5' overhangs were filled in using T4 DNA polymerase) was inserted. This was called pCREV. The EIAV gagpol reading frame including the RRE and leader was cut from pHORSE3.1 with *Xho* I and *Not* I (5.5kb) and inserted into pCREV at the *Xho* I and *Not* I sites to make pEGPR3.1.

Codon optimisation of the EIAV *gagpol* should eliminate the dependence of *gagpol* protein expression on the RRE/Rev system. The need of pEGASUS-1 for Rev/RRE can also be eliminated by using a heterologous RNA export system such as the constitutive transport element (CTE) from Mason-Pfizer Monkey virus (MPMV) (Bray et al., PNAS, 1994, 91:1256-1260, Kim et al., 1998)

Table 4

Vector	gagpol	Titre on D17 cells (l.f.u./ml)		Ratio (Non-dividing/dividing)
		Dividing	Non-dividing	
S2+	S2+, dUTPase+	2.2×10^5	1.1×10^5	0.5
S2-	S2+, dUTPase+	1.5×10^5	1.3×10^5	0.9
S2-	S2-, dUTPase+	1.0×10^5	1.2×10^5	1.2
S2-	S2-, dUTPase-	1.5×10^5	1.6×10^5	1.1
S2+	S2-, dUTPase+	2.2×10^5	2.3×10^5	1.0
S2+	S2-, dUTPase-	1.5×10^5	1.4×10^5	1.0
S2+	S2+, dUTPase-	1.5×10^5	1.4×10^5	1.0
MLV Vector		1.2×10^7	6.7×10^3	0.0006
Mock		< 1	< 1	1

Table 5.

Comparison of pONY2.10LacZ and pEGASUS +/- EIAV RRE.

Vector Genome	Gagpol	Titre (l.f.u./ml)
pONY2.10LacZ	pONY3.0	7×10^4
pEGASUS	pONY3.0	2.2×10^4
pEGASUS/RRE	pONY3.0	8.6×10^4

Titres with Rev are higher for pEGASUS-1 even though it has no RRE. Possibly the effect of REV is via enhanced expression of gagpol.

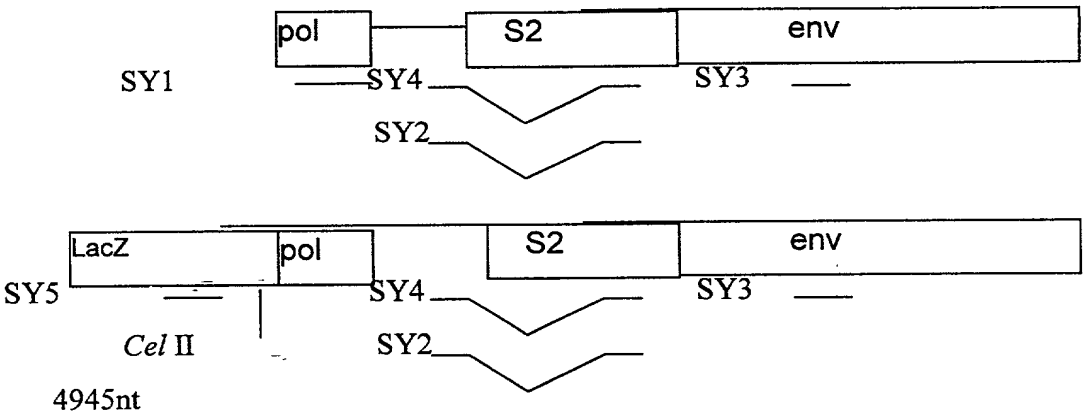
Table 6.

Vector Genome	Gagpol	Titre (l.f.u./ml)
pONY2.11lacZ	pONY3.1	1.7×10^5
pONY2.11lacZ	pHORSE3.1	9.0×10^4
pEGASUS/RRE	pONY3.1	8.0×10^4
pEGASUS/RRE	pHORSE3.1	2.0×10^4

Transfections were carried out in 293T cells with pCI-Rev and pRV67. The virus was titred on D17 cells

Primers

pONY3DS2



SY1: GAC A CCATGG GAA GTA TTT ATC AC (*Nco* I underlined)

5345nt 5321nt

SY2: CCT GGG ATT CAT ATC AAA CCT TAT AAC AAA TAT TG

5406nt 5397nt

5623nt

SY3: TCC T GCTAAGC ATA ACA GAA AC (*Cel* II underlined)

5397nt 5415nt

SY4: GGT TTG ATAT GAA TCC CAG GGG GAA TCTC

5336nt 5345nt

SY5: ACCC CGTACG TCT TCC CGA GCG (*Sun* I underlined)

dUTPaseF: GTTATTAATTAATGGAGGAATAATTGAAGAAGGATATAC

(*Pac* I underlined)

(The base in bold is the single base change from T to A that inactivated the dUTPase).

dUTPaseR: TCTTCTGCAGGTCCTGATCCTTGCTTAGTGC

(*Pst* I underlined)

S2StopR: GACCATGTTACCCCTTTACCATTAACTCCC**T**AATATCAAAC

(The bases in bold are the base changes from T**ATG**G to T**TAG**G that remove the start codon of S2).

S2StopF: GTAAAGGGGTAACATGGTCAGCATCGCATTCTA**C**GGGGGAATCC

(The base in bold is the base change from T**ATG**G to T**ACG**G that remove the start codon of S2).

EGAGP5'OUTER: CCATGCACGTCTCGAGCCAGCATGGGAGACCCTTTGAC

(*Xho* I underlined)

EGAGP3'OUTER:

CGAGCTAGAGGTCGACTCAATTTGGTTTATTAGTAAC

(*Sal* I underlined)

EGAGPINNER3: GCAATGGAATGACATCCCTCAGCTGCCAGTCC

(*Pvu* II underlined)

EGAGPINNER5:

GGGATGTCATTCCATTGCCACCATGGGAAGTATTTATCACTA

(*Nco* I underlined)

Example 10 - pONY4 series of vectors

In order to eliminate the use of Tat for the transcription of the EIAV genome and increase the amount of full length transcript the EIAV U3 (5' LTR) was replaced with the HCMV enhancer/promoter as in the case of the pEGASUS vectors (Example 2).

Plasmid construction.

pONY2.11lacZ contains a deletion in *gag* such that only 373bp of the gag ORF remains. pONY4 was made by replacing the 5' LTR with the CMV LTR from pEGASUS-1. pEGASUS-1 was cut with *Bgl II/Xho* I releasing a 3.2kb fragment (containing the CMV LTR) which was inserted into pSP72 cut with *Bgl II/Xho I.* This construct was named pSPPEG213. This was cut with *Hpa I/Nar I* and the 1.3kb fragment (encompassing the CMV LTR) was inserted into pONY2.11lacZ cut with *Nae I/Nar I.* pONY4.1 contains a deletion (2.1kb) downstream of the lacZ gene (between the *Sfu I* and *Sal I* sites) such that *tat, S2, env, rev* and RRE, are either missing or severely truncated (Figure 11c). pONY4.1 was made by cutting it with *Sfu I/Sal I,* blunt-ended by Klenow polymerase and religated. pONY4G was made by replacing the lacZ gene of pONY4 (*Sac II/Kpn I* and then blunting with Klenow polymerase) with that of GFP from pEGFP-N1 (Clontech) (*Bam HI/Xba I* and then blunting with Klenow polymerase) as a blunt fragment.

Production and Assay of Vectors.

Vector stocks were generated by calcium-phosphate transfection of human kidney 293T cells plated on 10cm dishes with 16μg of vector plasmid, 16μg of gag-pol plasmid and 8ug of envelope plasmid. 36-48 h after transfection, supernatants were filtered (0.45μm) aliquoted and stored at -70°C. Concentrated vector preparations were made by ultracentrifugation of at 20 000 rpm (SW40Ti rotor) for 90 min, at 4 °C. The virus was resuspended in PBS for 3-4 h aliquoted and stored at -70 °C. Transduction was carried out in the presence of polybrene (8μg/ml). It was consistently observed that pONY2.11lacZ gave about 2 to 4 fold higher titres than the less deleted

pONY2.10lacZ. When U3 in the 5' LTR was replaced with the CMV enhancer /promoter as in pONY4 then titres increase a further 5 to 10 fold.

Example 11 - EIAV 'self-inactivating' vectors (SIN-vectors)

The expression of the transgene from EIAV vectors in particular cell types may be influenced by elements in the LTR's. To remove such elements SIN (Self Inactivating) vectors can be constructed however the precise configuration of the vector may be influenced by the requirement to maintain certain sequences necessary for efficient production of the vector (Mol Cell Biol 1996 Sep;16(9):4942-51. RNA structure is a determinant of poly(A) site recognition by cleavage and polyadenylation specificity factor. Graveley BR, Fleming ES, Gilmartin GM) (J Virol 1996 Mar;70(3):1612-7. A common mechanism for the enhancement of mRNA 3' processing by U3 sequences in two distantly related lentiviruses. Graveley BR, Gilmartin GM). In addition SIN vectors provide a way for eliminating the production of full length transcripts in transduced cells.

Two SIN vectors were made: one containing the putatively important sequences (for polyadenylation), located between the *Mlu I* and *Mun I* sites and one in which these sequences were deleted. The 5' border of the deletions was 112 bases from the 5' end of the U3 region of the 3'LTR. The structure of two SIN vectors is shown in Figure 12.

Deletions present in pONY4G.SIN-MLU and pONY4G.SIN-MUN vectors are indicated in dashed lines. Primers are shown in italic.

DNA sequences between nucleotides 7300 and 8079 (numbered according to EIAV clone pSPEAIV19, Accession No. U01866) were obtained using polymerase chain reaction amplification using pONY4G as template. The positive sense primer was ERRE3 and the negative primers for amplification were
SIN-MLU (C7143: GTCGAGC**ACGCGT**TTGCCTAGCAACATGAGCTAG (*MluI* site in bold) or

SIN-MUN (C7142: GTCGAGC**CAATTG**TTGCCTAGC

AACATGAGCTAG (*MunI* site in bold) where the underlined sequences are complimentary to nucleotides 8058 to 8079 (of pSPEIAV19). The PCR products were digested with *NspV* and either *MluI* or *MunI* respectively. These were then ligated into pONY4G prepared for ligation by digestion with *NspV* (*SfuI*) and either *MluI* (partial digestion) or *MunI* respectively.

Example 12 - EIAV vectors with reverse configuration internal promoter-reporter cassettes.

In EIAV vectors such as pONY4Z or pONY4G the internal CMV-reporter cassette is orientated so that transcription from the 5'LTR and the internal promoter are co-directional and the polyadenylation signal in the 3'LTR is used for transcripts from both promoters. An alternative configuration is achieved by reversing the internal promoter-reporter cassette, however a polyadenylation signal must be placed downstream of the cassette.

An example of this 'reverse orientation' vector was made as follows. pONY4Z was digested with *PstI* and the overhanging termini trimmed back with T4 DNA polymerase. This was then used as the 'vector' fragment in a ligation with the MluI to AseI fragment from pEGFP-C1 which contains sequences including the CMV-GFP-SV40 early mRNA polyA signal cassette. Prior to ligation this fragment was flush-ended with T4 DNA polymerase. The vector encoding plasmid was called pONY4Greverse.

Vector particles were recovered from pONY4Greverse by co-transfection with pONY3.1 and pRV67, which express EIAV gag/pol and VSV-G protein respectively. The titre on D17 canine cells from pONY4Greverse was 13-fold lower than from pONY4G vector recovered in parallel.

The lower titre of pONY4Greverse was probably due to interference between the CMV promoters which drive transcription of the genome and the GFP towards each

other however truncation of the genomic RNA by the SV40-derived polyadenylation signal present in the inserted CMV-GFP-polyA cassette could also have been a factor. An improved vector was made by replacing the polyadenylation signal of pONY4Greverse with the bovine growth hormone polyadenylation (BGHpA) signal. To make this improvement pONY4Greverse was digested with *BstAPI* and the ends flushed with T4 DNA polymerase, then cut with *PstI*. This'vector' fragment was then ligated to a DNA fragment representing the BGHpA which was prepared from pcDNA3.1+ (Invitrogen) by digestion with *SphI*, and then the ends blunted with T4 DNA polymerase, then digested with *PstI*.

Example 13 - Construction and use of poly.A signals containing introns

In the pONY vectors described here the polyadenylation signal used is that from EIAV. This is found in the 3' LTR at the border of R and U5. This signal may not be optimal because it is not of a consensus sequence (see Whitelaw and Proudfoot 1986 EMBO 5; 2915-2922 and Levitt *et al* 1989 Gen. and Dev. 3; 1019-1025 for description of consensus polyadenylation signal).

One method of improving the viral polyadenylation is to replace the 3' LTR poly A signal with that of a consensus/strong polyadenylation signal. By such a method the signal would now be optimal in the producer cell. However upon transduction this signal is lost because during replication, the 5' LTR is the source of the poly A signal (see *Retroviruses* 1998 CSH press (Ed. J.Coffin) for review of retroviral life cycle). One novel way of overcoming the problem (of no strong polyadenylation signal upon transduction) is to include the poly A signal in a manner as will now be outlined: The method is to use a 'split poly-A signal' where by an intron splits the aataaa motif from that of the essential g/u box. Such a signal has previously been used by Liu *et al* (1993 N.A.R 21;5256-5263) to demonstrate both that large gaps between the aataaa and the g/u box will disable the poly A signal and that the polyadenylation process preceeds splicing. By placing a split-polyA signal within the retroviral vector such a signal will not be functional until transduction of target cells. This is because polyadenylation preceeds splicing and as such the upstream split-polyA signal will not

be used during vector expression within the producer cell. Outlined in Figure 13 is a schematic representation of how such a retroviral vector, containing a split polyA signal, would function - both in producer and in transduced cells. First this Figure demonstrates that although there exists an upstream consensus polyadenylation signal, the initial vector transcripts are still polyadenylated at the usual 3' LTR using either a viral or other poly A signal as so desired. This is because although the upstream poly A signal is functional in the final vector genome, this signal is not read by the polyadenylation machinery because it is created only during intron removal and thus not present in the primary RNA transcript. Second, this figure demonstrates that upon transduction the resulting vector transcripts are now polyadenylated at the first signal; this being now a normal strong polyadenylation signal with no introns to distance the essential aataaa and g/u box.

There are a number of advantages to inclusion of such a split-poly A signal within a retroviral vector; these include the following:

(1) The use of strong non-viral based polyadenylation signal within the transduced cell will enhance gene expression within such cells.

(2) The use of such poly A signals upstream of the natural LTR (see Figure 13) based signals will, upon transduction, generate shorter RNA transcripts that contain less viral sequence at their 3' end and as such will not be able to undergo subsequent retroviral reverse transcription. Indeed if the desired gene is expressed from an internal promoter such as the CMV, rather than an LTR; the resulting transcript expressed in the transduced cell could be designed to contain no viral sequence at all (see Figure 3 A).

(3) Inclusion of such a signal upstream of the 3'LTR will mean expression of the RNA downstream to the split poly A signal will be limited only to the producer cell because such RNA will not be transcribed in the transduced cell. This will therefore restrict certain sequence expression (for example IRESneo; see Figure 14 B) to producer cells.

(4) The presence of an intron within the producer cell will help with nuclear export of vector RNA from the nucleus.

(5) Because upon transduction their now exists an internal functional poly A signal, the viral poly A signal in the 5' LTR (the one copied to the 3' position during reverse transcription) can be removed/ deleted if desired. This is of use for preventing the process of promoter-proximal polydenylation from the 5' LTR in the producer cell (see Scott and Imperiale 1997 (Mol. Cell. Biol. 17;2127-35) and thus encourage full length transcript production of the virus.

Example.

To demonstrate the use of such a signal in a retrovirus; the "split poly A signal" cassette is constructed as described in Figure 15; with the intronic sequence being derived from pCI (Promega). Once made this cassette is cloned into the pONY 4 GFP vector using the *Pst1* compatible unique sse8387 site of pONY4-GFP (see Figure 16). Upon transduction the resulting vector will now polyadenylate prior to the 3'LTR and consequently no viral RNA 3' to lacZ will be transcribed (see Figure 16).

Example 14 - Construction of MLV/EIAV vectors

By replacing the EIAV LTR sequences with the MLV equivalents, the pONY vectors will no longer possess functional tar elements within the repeat regions (R) and as a consequence the U3 promoter will function without the requirement of Tat in the transduced cell.

Outlined in Figure 17 is how such a vector is made by overlapping PCR with primers described in Figure 18. Primers Me1 and Me2 are used to amplify a PCR product from the MLV vector pHIT111 (Soneoka *et al* 1995 NAR 23;628-633) whilst Me3 and Me4 are used to amplify a product from pONY4 lacZ. The resulting two products are then combined in a primerless PCR reaction to overlap them (homology between the two products is shaded in Figure 17). The final full length product is cut *BglII* and

XbaI and used to replace the *BglII-XbaI* fragment of pONY4 lacZ (containing the CMV/R/U5) to make pONY4-5'MLV. The resulting vector now has the CMV/R/U5 sequence from MLV linked to the EIAV U5 sequence (sequence required for genome recognition by intergrase prior to intergration). The next step involves PCR amplification with primers Me5 and Me6 from pONY4 LacZ template and PCR amplification with Me7 and Me8 from pLXSN template (Miller and Rosman 1989 Biotechniques 7:980-990) . These two PCR products are then overlapped by primerless PCR (homology between primers shown as hatched box) and the resulting fragment cut with *NspV* and *MunI* and inserted into the *NspV/MunI* sites of pONY4-5'MLV; thus replacing the 3'EIAV LTR with a 3'MLV LTR fused to the 3'UTR/ppt/U3 intergrase binding site of pONY 4 lacZ. The resulting plasmid, named pONY-MOUSE (see Figure 19 for complete DNA sequence), titres at 10^{4-5} per ml when combined with pONY3.1 and pRV67 in the HIT system.

Example 15 - Early promoter driving lentiviral vector genome

In this example an EIAV genome is expressed from a vaccinia early promoter P7.5E (Davison 1989a). The promoter has been engineered to produce an EIAV genome with the correct 5' RNA end. In addition the vaccinia early termination sequence has been inserted downstream of the EIAV genome. This is inserted into the transfer plasmid pSC65, which can homologously recombine into the TK region of the MVA genome. Recombinant viruses can be selected by their lack of sensitivity to BudR (Earl et al. 1998).

Figure 11 is a schematic representation of the EIAV genome vectors pONY4.0 and pONY4.1 which have been described in Example 10 and the vaccinia transfer vector pSC65 (Chakrabarti et al 1997). The P7.5E sequence is AAAAGTAGAAAATATATTCTAATTTATT. The Early termination sequence for the early promoters is TTTTTNT (N= any nucleotide) (Fields).

The DNA manipulations are as follows and Figures 20 and 21 give the sequence of the PCR primers. PCR with primers EMVA1/2 produces the 5' LTR with the U3

region replaced by the P7.5E promoter. This is inserted into the plasmid pSP72 (Promega) using the *Hind III/Pst I* sites to make pEMVA1. EIAV U3 contains a sequence matching the criteria for vaccinia early termination (TTTTTAT). Using primers EMVA3/4 and EMVA5/6 and overlapping PCR this region is mutated to TTTCCAT in order to prevent early termination. This PCR product is inserted into the pEMVA1 using the *Bgl II/Pst I* sites to generate pEMVA2. A termination sequence (TTTTTTTTT) is inserted downstream of the 3' LTR R region using primers EMVA7/8. This PCR product is inserted into pEMVA2 using the *Mun I/Bgl II* sites making pEMVA3. Into this plasmid the rest of the EIAV vector genome (pONY4) is inserted via the *Nar I/Nsp V* sites making pEMVA4 (Figure 22). This is then cut with *Pac I/Bgl II* and inserted into pSC65 cut with *Pac I/Bam HI* to make pEPONY4 (*Bgl II* and *Bam HI* are compatible) (Figure 23). This removes the two vaccinia promoters and the lacZ coding cassette from pSC65.

In order to make the minimal EIAV genome version of this construct that is analogous to pONY4.1, pEMVA4 is cut with Sal I/Nsp V blunt ended and religated to make pEMVA5 (Figure 24). This removes much of the sequence between the end of the lac Z gene and the end envelope region, hence this vector is Tat, Rev, S2 and Env minus. This is described in Example 10. This is then cut with *Pac I/Bgl II* and inserted into pSC65 cut with *Pac I/Bam HI* to make pEPONY4.1 (*Bgl II* and *Bam HI* are compatible) (Figure 24). This removes the two vaccinia promoters and the lacZ coding cassette from pSC65.

Both pEPONY4.0 and pEPONY4.1 are suitable for inserting the genome expression cassettes into the TK region of the MVA genome (Carroll MW and Moss B Virology 1997 Nov 24;238(2):198-211) using a BHK TK-ve cell line (ECACC 85011423) and standard procedures for the construction of recombinant poxviruses (Earl et al 1998a & 1998b)

Example 16 – Synthetic early/late promoter driving lentiviral vector genome

The synthetic early/late promoter of vaccinia has a requirement for sequences downstream of the RNA initiation site (Davison 1989b). For this promoter to be used to generate a retroviral genome either the R regions have to be modified or a ribozyme is used to make the correct 5' end. Modifying the R regions is problematic as the initiation site has not been conclusively identified and varies with the sequence (Davison 1989b). Below is described the generation of a transfer plasmid that expresses the EIAV genome from the synthetic early/late promoter (Psyn). Downstream of this promoter is inserted a ribozyme that ensures the creation of the correct 5' end of the RNA. This construct also contains the early termination sequence.

The DNA manipulations are as follows: PCR with primers EMVA9/1 produces the 5' LTR with the U3 region replaced by the Psyn promoter and a hammerhead ribozyme (Figure 25 and 26). This is inserted into the plasmid pEMVA4 (Example 15) using the *Pac I/Nar I* sites to make pEMVA6 (Figure 27). This is then cut with *Pac I/Bgl II* and inserted into pSC65 cut with *Pac I/Bam HI* to make pSynPONY4 (*Bgl II* and *Bam HI* are compatible) (Figure 27). This removes the two vaccinia promoters and the lacZ coding cassette from pSC65.

In order to make the minimal EIAV genome version of this construct that is analogous to pONY4.1, pEMVA6 is cut with *Sal I/Nsp V* blunt ended and religated to make pEMVA7 (Figure 28). This is then cut with *Pac I/Bgl II* and inserted into pSC65 (a vaccinia transfer vector) cut with *Pac I/Bam HI* to make pSynPONY4.1 (*Bgl II* and *Bam HI* are compatible) (Figure 28). This removes the two vaccinia promoters and the lacZ coding cassette from pSC65.

Both pSynPONY4.0 and pSynPONY4.1 are suitable for inserting the genome expression cassettes into the TK region of the MVA genome (Carroll MW and Moss B Virology 1997 Nov 24;238(2):198-211) using standard procedures for the construction of recombinant poxviruses (Earl et al 1998a & 1998b)

Example 17 – T7 promoter driving lentiviral vector genome

The T7 promoter can be used to generate a retroviral genome which can make the correct 5' end. Below is described the generation of a transfer plasmid that expresses the EIAV genome from the T7 promoter (T7). Downstream of this promoter is inserted a T7 termination sequence. This is inserted into the transfer plasmid pSC65, which can homologously recombine into the TK region of the MVA genome. The T7 promoter requires the T7 polymerase. MVA viruses are available which express T7 polymerase from Vaccinia promoters (Wyatt et al 1995).

The T7 promoter has the sequence (-)TAATACGACTCACTATAGG(+2) with transcription beginning after A with preferably a run of Gs. The T7 termination sequence is CTAGCATAACCCCTTGGGGCCTCTAAACGGGTCTTGAGGGGTTTTTTG. The T7 promoter and terminator sequences are as those described in the plasmid pCITE-4a(+) (Novagen).

The DNA manipulations are as follows. PCR with primers EMVA10/11 (Figure 29) produces the 5' LTR with the U3 region replaced by the T7 promoter. This is inserted into the plasmid pEMVA4 using the *Pac I/Nar I* sites to make pEMVA8 (Figure 30). PCR with primers EMVA11/7 produces part of the 3' LTR with a T7 termination sequence. This is inserted into pEMVA8 using the *Mun I/Bgl II* sites to make pEMVA9. This is then cut with *Pac I/Bgl II* and inserted into pSC65 (a vaccinia transfer vector) cut with *Pac I/Bam HI* to make pT7PONY4 *(Bgl II* and *Bam HI* are compatible) (Figure 30). This removes the two vaccinia promoters and the lacZ coding cassette from pSC65.

To make the minimal EIAV genome version of this construct (pONY4.1) pEMVA9 is cut with *Sal I/Nsp V* blunt ended and religated to make pEMVA10 (Figure 31). This is then cut with *Pac I/Bgl II* and inserted into pSC65 (a vaccinia transfer vector) cut with *Pac I/Bam HI* to make pT7PONY4.1 (*Bgl II* and *Bam HI* are compatible) (Figure 31).

This removes the two vaccinia promoters and the lacZ coding cassette from pSC65.

Both pT7PONY4.0 and pT7PONY4.1 are suitable for inserting the genome expression cassettes into into the TK region of the MVA genome (Carroll MW and Moss B Virology 1997) using standard procedures for the construction of recombinant poxviruses (Earl et al 1998a & 1998b).

Example 18 – Construction of an EIAV gag/pol cassette for expression in vaccinia.

Normally EIAV gag/pol requires Rev/RRE for expression as Rev enables the unspliced transcript to be exported out of the nucleus. As Pox viruses are cytoplasmic, EIAV viral RNA export should not be a problem. But if Rev has other functions such as RNA stability or acts as a translation enhancer it can be expressed in a similar way to EIAV *gag/pol* (Martarano 1994). Alternatively the EIAV *gag/pol* sequence can be codon optimised to overcome the Rev /RRE requirement for export and enhance RNA stability. Below is described the creation of a vector that expresses EIAV gag/pol from a synthetic early/late promoter (Psyn). This is inserted into the transfer plasmid pLW-22 (Wyatt and Moss Appendix 1, Earl et al 1998a & b), which can homologously recombine into the Del II region of the MVA genome. Recombinant viruses can be selected by their expression of lac Z.

EIAV *gag/pol* including the leader the gag/pol open reading frame and the RRE can be obtained from cutting pHORSE3.1 (Example 9) with *Xho I/Not I* to give a 5.5kb band (Figure 32). This is then inserted into the vaccinia transfer vector pLW-22 cut with *Sal I/Not I* (*Sal I* and *Xho I* are compatible) to make pLWHORSE3.1 (Figure 32).

Example 19 – Construction of an EIAV rev cassette for expression in vaccinia

In the event that Rev is required for EIAV viral vector production from a poxvirus it can be expressed from a synthetic early/late promoter. This construct is inserted into the transfer plasmid pMC03, which can homologously recombine into the *Del III*

region of the MVA genome. Recombinant viruses can be selected by their expression of GUS (Carroll et al. 1995).

The DNA manipulations are as follows. Plasmid pCIRev is described in Example 9. It is an EIAV Rev expression plasmid. This is cut with *Afl II/Not I* (0.6kb), blunt ended by T4 DNA polymerase and inserted into pMC03 (Carroll et al. 1995) cut with *Pme I* to make pMCRev (Figure 33).

Example 20 – Construction of heterologous envelope cassettes for expression in vaccinia

EIAV can be pseudotyped with a number of envelopes such as VSV-G and amphotropic MLV envelope. Below is described the creation of a MVA transfer vector that expresses the amphotropic envelope or VSV-G envelope from the P7.5 early/late promoter. The transfer vector is pYF6 which can homologously recombine into the HA region of MVA. Recombinant viruses can be selected by direct live immunostaining for expression of the env.

In order to produce a transfer vector containing a VSV-G cassette, the VSV-G expression plasmid pRV67 (Kim et al. 1998) is cut with *Sma I/Eco RV* (1.7kb) and the resulting fragment inserted into pYF6 cut with Sma I to make pYFVSVG (Figure 34). Similarly, to produce an analogous amphotropic envelope construct pHIT456 (Soneoka 1995) is cut with *Xba I* and the 2.2kb band blunt ended by T4 DNA polymerase and inserted into pYF6 cut with *Sma I* making pYFAmpho (Figure 35).

Both pYFAmpho and pYFVSVG are suitable for inserting the genome expression cassettes into into the HA region of the MVA genome using standard procedures for the construction of recombinant poxviruses (Earl et al 1998a & b, Flexner et al 1987)

Example 21 - Construction and Amplification of MVA-Lenti Recombinants

The recombinant vaccinia viruses containing multiple inserts encoding the components of the EIAV vectors (Figure 25) are constructed by sequential recombination with the relevant transfer plasmids. The construction of v.MEeG-0r (Figure 36) is used as an example:

1. A plasmid carrying gag-pol (pLWHORSE3.1) is transfected into BHK-21 or CEF cells, that have been previously infected with MVA (as described in Carroll and Moss 1997, Earl et al 1998a & b).
2. After two days of infection recombinant MVA virus is assayed on BHK-21/CEF and cells are over-layed with agar medium containing the substrate for the colour marker β-galactosidase (Chakrabarti et al 1985) which is expressed from within pLW22.
3. Blue plaques are picked and plaque purified until a homogeneous recombinant virus population is obtained.
4. Recombinant virus is then used to recombine with transfer plasmids containing the other recombinant genes: pMCRev in which selection is based on GUS expression (Carroll & Moss 1995), the genome (pEPONY4.0) in which selection is based on a TK negative phenotype using BudR (Carroll & Moss 1997, Earl et al 1998a & b) and VSVG (pYFVSVG) in which recombinants are identified by direct immunostaining of VSV G (Earl et al 1998a & b).

Recombinant viruses may be amplified in BHK-21 or CEF cells as described below:

Propagation of Vaccinia Virus

The highly attenuated strain MVA is derived from the replication competent strain Ankara and has endured over 570 passages in primary chick embryo fibroblast cells. MVA replication was initially thought to be restricted to CEF cells as only minimal replication in mammalian cells was reported. However, further analysis has shown that Baby Hamster Kidney cells (BHK-21) are able to support high titre production of MVA. MVA may thus be grown on BHK-21 or primary CEF cells

(Carroll & Moss (1997) Virology **238**:198- 211).

To prepare CEF cells, 10 day old chick embryos are gutted and limbs and head are removed before being minced and trypsinised in a solution of 0.25% trypsin and incubation at 37^{0}C . The cell suspension is filtered through a course filter mesh and cells are washed and concentrated by centrifugation at 2000 rpm in a Sorvall RC-3B at 1500 rpm for 5 mins. Cells are suspended in MEM containing 10% FCS, aliquotted into 175cm flasks and incubated at 37^{0}C in a CO_2 incubator. When monolayers are 95% confluent they are trypsinised and used to seed additional flasks or six well plates. Alternatively, primary cultures are transferred to a 31^{0}C incubator for later use (Sutter and Moss (1992) Proc Natl Acad Sci U S A **89**:10847-10851).

Preparation of crude, semi-purified and purified virus stocks

Crude virus stocks are prepared for initial recombinant virus analysis or as viral stocks used for subsequent high titre virus preparations. Vaccinia virus preparations can be semi-purified by centrifuging out cell membranes and nuclei or by additional steps involving sucrose centrifugation to prevent contamination by pre-expressed recombinant protein products and cellular organelles. Methods used are a modification of those described by Earl *et al.,* 1998a & b; Earl and Moss, *ibid*, pp. 16.17.1-16.17.16; Earl and Moss, *ibid*, pp. 16.18.1-16.18.10; and Bronte *et al.*, (1997) Proc Natl Acad Sci U S A 94(7):3183-3188.

Crude Virus

MVA is grown in either CEF or BHK-21 (obtained from the ATCC) and WR is grown in HeLa or BS-C-1 (ATCC) in 175cm^2 tissue culture flasks. Briefly, confluent monolayers are infected with an moi of approx. 1 pfu with MVA or WR. Virus is suspended in 10ml MEM containing 2% FCS and added to 175cm^2 flasks containing confluent cell monolayers. After inoculation for 1 hour at 37^{0}C an additional 20ml MEM containing 2% FCS is added. After 48-72 hours infected cells are scraped into the medium and pelleted at 1500g for 5 mins. For crude virus preparations cells are resuspended 2ml MEM (2%) per 175cm^2 flask. Cells are freeze thawed three times, sonicated and aliquotted into 1 ml freezing tubes. A representative aliquot is freeze

thawed and titred to determine virus concentration. Virus stocks are stored below -20°C.

Semi-pure preparations

Infected cells are harvested as described previously (Earl *et al* a & b; Earl and Moss; 1991). After centrifugation cells are resuspended in PBS (2ml/175cm^2 flask) and homogenised by 30-40 strokes in a tight fitting glass dounce homogeniser, on ice. Cell breakage is checked by microscopy. Nuclei, cellular organelles and membranes are removed by a centrifugation at 300g for 5 mins (4^{0}C), keep supernatant. The cell pellet is resuspended in 1ml/175cm^2 flask and centrifugation repeated. The supernatants are pooled, aliquoted and stored.

Purified preparation

Infected cells are harvested as previously described (Earl *et al.*a & b; Earl and Moss; 1991) and resuspended in 10 mM Tris.Cl, pH 9.0 (2ml/flask), keeping samples on ice from this point of the procedure. Homogenise as described previously using 10 mM Tris. The lysate is sonicated (on ice) using an XL 2015 sonicating cup (Misonics, USA) at maximum output (500 W) for 1 min. The sample is placed on ice for 1 min and the sonication repeated up to 3 times. A maximum of 5ml is sonicated at a time, and ice is replenished during sonication. The lysate is gently layered onto a cushion of 17 ml of 36% sucrose (in 10 mM Tris.Cl, pH 9.0) in a SW-27 centrifuge tube. Lyates are centrifuged for 80 mins in an SW-27 rotor at 13 500 rpm (32,900 x g), 4°C. The supernatant is discarded and the viral pellet resuspended in sterile PBS and sonicated in a cup sonicator for 1 min (on ice). Concentrated virus is aliquoted and stored at below -20^{0}C.

Example 22 - Production of EIAV vector particles from MVA-EIAV Hybrids

As described above large scale preparations of recombinant MVA-EIAV are made. These preparations are used to infect mammalian cells that are non-permissive for MVA, such that the resulting supernatant will only contain EIAV and not infectious MVA (Meyer et al 1991, Carroll and Moss 1997). A suitable cell line is MRC5 (ATCC). Cells are infected at an MOI of 3. Infections are allowed to run for

approximately 48 hours before supernatants are harvested and EIAV vector particles either used directly or concentrated/purified by ultracentrifugation or cross-flow methods. To produce large scale preparations, are grown in suspension or on microcarriers or in roller bottles. EIAV vectors carrying gene of interest prepared in these ways are used to transduce target cells in vivo or in vitro.

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